
Electronic Thesis and Dissertation Repository

9-13-2013 12:00 AM

ER stress coupled pannexin channel activation via STIM proteins

Ankur Bodalia

The University of Western Ontario

Supervisor

Dr. Michael F. Jackson

The University of Western Ontario Joint Supervisor

Dr. John F. MacDonald

The University of Western Ontario

Graduate Program in Pharmacology and Toxicology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

© Ankur Bodalia 2013

Follow this and additional works at: <https://ir.lib.uwo.ca/etd>



Part of the [Medical Pharmacology Commons](#), [Medical Physiology Commons](#), and the [Neurosciences Commons](#)

Recommended Citation

Bodalia, Ankur, "ER stress coupled pannexin channel activation via STIM proteins" (2013). *Electronic Thesis and Dissertation Repository*. 1623.

<https://ir.lib.uwo.ca/etd/1623>

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlsadmin@uwo.ca.

ER STRESS COUPLED PANNEXIN CHANNEL ACTIVATION
VIA STIM PROTEINS

(Thesis format: Monograph)

by

Ankur Bodalia

Graduate Program in Pharmacology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

© Ankur Bodalia 2013

Abstract

Many neurodegenerative diseases, including Alzheimer's Disease, are associated with ER stress. It is suggested that plasma membrane channels contribute to the increased ionic influx and subsequent cell death in response to ER stress. Pannexin channels, which have been implicated in various pathophysiological conditions, are a suitable candidate for facilitating this response. However, mechanisms of pannexin channel activation are poorly defined. I investigated the potential regulation of pannexin activity by the ER stress sensor, STIM. It was hypothesized that pannexin channel activation during ER stress is contingent on the recruitment of STIM proteins. In neurons, pannexin activation was observed in response to pharmacological ER stressors as well as A β ₄₂. Moreover, I identified STIM as a key signaling component promoting pannexin activation under ER stress in HEK cells, though not in neurons. In conclusion, pannexin activation is coupled to ER stress in neurons; however, the underlying molecular mechanism remains unknown.

Keywords

pannexin, ER stress, STIM proteins, calcium, β -amyloid, electrophysiology

Co-Authorship Statement

Dr. Michael F. Jackson supervised and contributed to the design of all experiments discussed in this work. Dr. Hongbin Li conducted preliminary electrophysiology experiments assessing pannexin channel activation in response to various ER stress inducing compounds (Appendix A S1A) and pharmacologically identifying the evoked currents as emanating from pannexin channels (Appendix A S12). Natalie Lavine performed Western blot of beta-amyloid₁₋₄₂ to test for oligomerization (Appendix A S2). Dr. Hongbin Li contributed electrophysiological data to the initial recordings of pannexin currents in HEK cells transfected with STIM (Appendix A S3). Natalie Lavine performed Western blots on sister cultures to the infected neurons where STIM expression was knockdown (Appendix A S4).

Acknowledgments

First and foremost, Dr. Mike Jackson, for always being patient with me. All graduate students have a learning curve and it takes a great mentor to not only be understanding of this, but to work with the student to overcome their challenges and realize their potential. Electrophysiology came with its fair share of challenges but Mike Jackson continued to sit with me at the rig and guide me through the art of patch clamping. From preparing presentations to thesis writing, he was not only patient with me but also supportive and advising every step of the way. He was also instrumental in my search for a new lab and despite his move to Winnipeg, he was still there to support me through to the end. Thank you, Mike, for being an amazing supervisor, mentor and friend.

Secondly, Dr. John MacDonald, I thank you for giving me the opportunity to be a part of the MacDonald-Lab. Despite being very highly regarded by your peers, you remain very humble, a trait I admire. The way you've conducted yourself during the more difficult times has been inspiring to all the lab members.

My advisory committee, Dr. Wei-Yang Lu, Marco Prado and Rommel Tirona, for their advice and guidance along the way. Dr. Lu, thank you for challenging me to do my best when faced with obstacles - I owe you one!

I appreciate the comradery of all the lab colleagues. Despite the bi-weekly barrage of scientific criticism, even Dr. Kai Yang contributed to my development by preparing for the toughest of judges. A special thank you to Natalie Lavine, ie. the Mr. Miyagi of biochemistry, for teaching this young grasshopper the value of a well-maintained cell culture room.

Outside the lab, I thank Dr. Andrew Watson for his mentoring, motivation and killer reference letters! I would also like to thank Dr. David Freeman for giving me my first gig in research. Under your training, I learned that research is like playing detective, putting evidence together to solve a biological mystery. The more you uncover, the more curiosity you spark.

This section wouldn't be complete without a thank you to my friends and family for their encouragement from day one.

Finally, a shout-out to the gang from Woodward Whisky Wednesdays #OnTheRocks

Table of Contents

Abstract.....	ii
Co-Authorship Statement.....	iii
Acknowledgments.....	iv
Table of Contents.....	v
List of Tables.....	vii
List of Figures.....	viii
List of Appendices.....	ix
List of Abbreviations.....	x
 Section 1: Introduction.....	 1
1.1 Overview of ER stress in neurodegenerative diseases.....	2
1.2 Pannexin channel structure and function.....	3
1.3 Functional identification of pannexin activity.....	5
1.4 Regulation of Ca ²⁺ homeostasis by the ER.....	8
1.5 Cellular responses to ER stress.....	9
1.6 Store-operated Ca ²⁺ entry, Stromal Interacting Molecules.....	10
1.7 STIM structure and function.....	11
1.8 ER stress in Alzheimer's Disease, beta-amyloid.....	15
1.9 Rationale and hypothesis.....	16
 Section 2: Materials and Methods.....	 18
2.1 Preparation of primary cultured mouse hippocampal neurons.....	19
2.2 Preparation of pannexin and STIM expressing HEK 293 cells.....	19
2.3 Preparation and application of A β ₄₂	22
2.4 Electrophysiology: whole-cell patch clamp recordings.....	22
2.4.1 Recording acute-response pannexin currents.....	26
2.4.2 Recording chronic-response pannexin currents.....	27
2.5 Statistical analysis.....	27
2.6 Sources of drugs, reagents and peptides.....	28
 Section 3: Results.....	 29
3.1 ER stress induces pannexin activation.....	30
3.2 A β ₄₂ induced pannexin activation in neurons.....	33
3.3 ER stress induced pannexin-1 activity is modulated by STIM proteins.....	37
3.4 ER stress-pannexin current is not STIM-dependent in neurons.....	41
3.5 ER stress-pannexin current is not Src-kinase-dependent in neurons.....	45
3.6 ER stress-pannexin current is not caspase-dependent in neurons.....	48
 Section 4: Discussion.....	 50
4.1 Summary of key findings.....	51
4.2 Pannexin channel activation in response to ER stress.....	51
4.3 ER stress-pannexin coupling modeled in neurodegenerative disease.....	53

4.4	Molecular basis for pannexin activation during ER stress.....	55
4.5	Contribution of STIM to pannexin channel activation in neurons.....	58
4.6	Exploring alternative mechanisms of pannexin activation in neurons.....	60
4.7	Experiments for future studies.....	63
4.8	Significance of the study.....	66
Section 5: References.....		67
Appendices.....		76
Appendix A: Supplementary Figures.....		77
Appendix B: Curriculum Vitae.....		81

List of Tables

Table 1.1:	Pharmacological profile of candidate non-selective cation channels.....	7
------------	---	---

List of Figures

Figure 1.1:	Proposed model of ER stress coupled pannexin activation, mediated by STIM proteins.....	14
Figure 2.1:	Representation of the full length STIM1 protein and the ERM domain deletion in STIM1 _{ΔERM} variant.....	21
Figure 2.2:	Images of cells during patch-clamp experiments.....	25
Figure 2.3:	Representation of extracellular solution application via a shifting barrel perfusion system in close proximity to the patched neuron.....	26
Figure 3.1:	Pharmacological ER stress inducers evoke pannexin activation in hippocampal CA1 neurons.....	32
Figure 3.2:	Effect of 24 hour extracellular pre-treatment of 1 μM Aβ ₄₂ oligomers on pannexin currents in neurons.....	35
Figure 3.3:	Effect of ERM-domain deletion from full-length STIM on ER stress induced pannexin-1 currents in HEK cells.....	39
Figure 3.4:	Effect of STIM1 & 2 knockdown on ER stress pannexin currents in neurons.....	43
Figure 3.5:	Effect of Src-family kinase inhibition on ER stress induced pannexin currents in neurons.....	47
Figure 3.6:	Effect of caspase inhibition on ER stress induced pannexin currents in neurons.....	49
Figure 4.1:	Schematic model of STIM-ORAI interaction during store-operated Ca ²⁺ -entry.....	56

List of Appendices

Appendix A: Supplementary Figures.....	77
Appendix B: Curriculum Vitae.....	81

List of Abbreviations

A β	beta-amyloid
AD	Alzheimer's disease
ATF6	activating transcription factor-6
ATP	adenosine triphosphate
BiP	binding immunoglobulin protein
CA1	Cornu Ammonis 1 region
Ca ²⁺	calcium
CAD	CRAC-activating domain
CBX	carbenoxolone
CCE	capacitive calcium entry
CHOP	CCAAT/-enhancer-binding protein homologous protein
CNS	central nervous system
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CPA	cyclopiazonic acid
CRAC	calcium release activated calcium channel
D-MEM	Dulbecco's modified eagle medium
DIV	days <i>in vitro</i>
ECF	extracellular fluid
EGTA	ethylene glycol tetraacetic acid
ER	endoplasmic reticulum
ER-PM	endoplasmic reticulum-plasma membrane
ERM	ezrin/radixin/moesin

FBS	fetal bovine serum
FFA	flufenamic acid
GFP	green fluorescence protein
HEK 293	human embryonic kidney 293
HFIP	hexaflouro-2-propanol
IC ₅₀	half-maximal inhibitory concentration
ICF	intracellular fluid
IP ₃ R	inositol triphosphate receptor
IRE1	inositol requiring enzme 1
I-V	current-voltage
kDA	kilo-Dalton
La ³⁺	lanthanum
mPannx1	mouse pannexin-1
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na ⁺	sodium
NMDA	N-methyl-D-aspartate
NMDAR	NMDA Receptor
PERK	double-stranded RNA-activated protein kinase-like ER kinase
PRB	probenecid
PSD-95	post-synaptic density 95
ROS	reactive oxygen species
RNAi	RNA interference
RNS	reactive nitrogen species

SAM	sterile- α -motif
SERCA	sarcoplasmic endoplasmic reticulum ATPase
shRNA	short hairpin RNA
SOCE	store-operated calcium entry
STIM	stromal interacting molecule
TEA	tetraethylammonium
Tg	thapsigargin
Tn	tunicamycin
TRPC	Transient Receptor Potential Cation channel
TRPM2	Transient Receptor Potential Melastatin Channel
TTX	tetrodotoxin
UPR	Unfolded Protein Response
¹⁰ panx	pannexin-inhibitory peptide

Section 1

INTRODUCTION

1.1 Overview of ER stress in neurodegenerative diseases

Many neurodegenerative diseases, including Alzheimer's Disease (AD), are associated with the misfolding of proteins, calcium (Ca^{2+}) dysregulation and resultant apoptotic cell death. Whether these pathological processes are interrelated has remained an open question. Within a neuron, the organelle responsible for protein folding, and an important regulator of Ca^{2+} homeostasis, is the endoplasmic reticulum (ER). In the context of neurodegenerative diseases, the ER has been shown to have exhausted its capacity to regulate Ca^{2+} homeostasis leading to an accumulation of misfolded and unfolded proteins otherwise known as ER stress. ER stress markers have been found to be up-regulated at early stages in AD patients. Moreover, ER stress has been proposed to contribute to β -amyloid₁₋₄₂-induced neurotoxicity, however the contributing mechanisms have not been precisely defined. Pannexin, a large-pore channel soluble to solutes of up to 1 kDa, has been implicated in neuronal cell death and is a suitable candidate for studying ER stress mediated ionic influx. Moreover, a recent study has provided evidence suggesting that $\text{A}\beta_{42}$ promotes activation of a large-pore channel, though its identity was not confirmed. Evidence from the Jackson/MacDonald labs suggests that ER stress is associated with pannexin channel opening. This led us to propose that ER stress may be coupled to pannexin opening with $\text{A}\beta_{42}$ treatment. In addition, as pannexin activation mechanisms are poorly understood, I investigated mechanisms by which neuronal ER stress is coupled to pannexin activation.

1.2 Pannexin channel structure and function

The pannexin channel is a large-pore non-selective ion channel located on the plasma membrane of neurons. Although pannexin channels were previously reported to localize to the ER (Penuela et al., 2007), those particular variants of pannexin have been identified as incomplete-glycosylation forms of pannexin, which fail to escape the ER for trafficking to the cell surface (Bhalla-Gehi et al., 2010). The ER-retained forms of pannexin are thought to be destined for premature degradation by proteosomes, a view that is supported by its co-localization to calnexin, an ER chaperone that retains misfolded proteins for premature degradation (Gehi et al., 2011). Rather than functioning as gap junctional hemichannels, pannexins operate individually as *bona fide* plasma membrane channels connecting cytoplasm to the extracellular space (MacVicar and Thompson, 2010). Pannexin-1, first cloned in 2003, is the only ubiquitously expressed member of the pannexin family (Bruzzone et al., 2003). Pannexin-2 expression is predominantly confined to tissues of the central nervous system, whereas pannexin-3 is expressed in skin and bone (Baranova et al., 2004). The ability of pannexin-2 and pannexin-3 to form functional channels on their own has been debated, however when co-expressed with pannexin-1, heteromeric channels with their own distinct properties have been generated (Penuela et al., 2009). Of the three, pannexin-1 is the most studied to date and the focal point of my experiments.

Structurally, pannexin channels contain 4-transmembrane domains, an intracellular loop, intracellular N and C termini and highly conserved cysteine residues on two extracellular loops (Bruzzone et al., 2003). The original view that pannexins functioned as gap junction hemichannels stemmed from their topological similarities to the gap junctional

Innexin protein family found in invertebrates (Phelan and Starich, 2001). Pannexins have since been proven not to be gap junctional proteins based on the following conclusive evidence from various labs:

- In a paired oocyte assay, transjunctional currents are prominent in connexin expressing pairs but absent in pannexin expressing pairs (Dahl et al., 1992; Bruzzone et al., 2003)
- Pannexin-1 expression has been observed in single non-gap junction forming cells such as erythrocytes (Locovei et al., 2006)
- In polarized non-symmetrical cells, pannexin-1 is found exclusively on the apical membrane which is not involved in cell-to-cell contact (Ransford et al., 2009)
- In neurons, pannexin-1 is found exclusively at post-synaptic membranes and shows no sign of involvement in electrical synapses (Zoidl et al., 2007)

With this evidence, pannexins are now widely accepted as large pore forming channels in the plasma membrane. A single pannexin channel is a hexamer of six subunits that allows non-selective permeation to ions and small molecules up to 1 kDa in size (Boassa et al., 2007; Ambrosi et al., 2010). This large pore size and non-selective permeability describes the unique nature of pannexin channels. When describing ion channel selectivity, channels that are permeable to both cations and anions, pannexin channels for instance, are considered “non-selective”. In addition to ion transport, pannexin channels have also been shown to release large metabolites such as ATP, as well as interleukins and glutamate (Bao et al., 2004; Jiang et al., 2007). Two important studies have functionally classified pannexin channels as ATP-release channels, however, an upstream trigger for pannexin opening remains undetermined. ATP release from pannexin-1

channels serves to induce vasodilation in erythrocytes under hypoxic conditions (Locovei et al., 2006). Additionally, the same function was noted in apoptotic lymphocytes where the observed ATP release from pannexin channels serves as a “find me” signal for macrophages (Chekeni et al., 2010). Given that ER stress markers are elevated in both apoptosis (Galehdar et al., 2010) and hypoxia (Tajiri et al, 2004), an ER stress triggered opening of pannexin channels could be a plausible mechanism underlying these observations.

1.3 Functional identification of pannexin activity

From reviewing literature on pannexin characterization, identification of pannexin-1 channel activity involves a combination of biochemical assays, DNA overexpression/silencing, pharmacological inhibition and electrophysiological recordings. In addition to the main conductance state, four different sub-conductance states have been observed in single channel recordings from excised patches in pannexin-1 expressing oocytes (Bao et al., 2004). Though not entirely agreed upon, some groups have characterized pannexin-1 whole cell currents as outward rectifying, activated at increasingly depolarized potentials (Ma et al., 2009). In this study, to reliably identify pannexin-1 channel activity and critical mediators in the activation pathway, I have paired RNA interference (RNAi) and DNA over-expression techniques with pharmacological characterization during electrophysiological recording of pannexin-1 activity.

Of the variety of known pharmacological inhibitors for pannexin-1, some of these

compounds overlap in their ability to block various non-selective cation channels (Table 1). For example, pannexin-1 blocker carbenoxolone is also known to block connexin hemichannels. Additionally, SKF96395, which does not block pannexin-1 channels, is known to block ORAI and TRPC channels. These inhibitors, among others, are utilized to pharmacologically identify pannexin-1 currents using a combinatorial approach to rule-out connexin hemichannels and other non-selective cation channels but rule-in pannexin channels based on their differential sensitivity to blockers. From the literature, pannexin-1 activity is inhibited by carbenoxolone (cbx) with an IC_{50} of 5 μ M, and probenecid (prb) with an IC_{50} of 150 μ M. Contrastingly, pannexin-1 is relatively insensitive to flufenamic acid (ffa) with an IC_{50} = 300 μ M while connexins are more sensitive to inhibition by ffa with an IC_{50} of 3-100 μ M but also relatively insensitive to cbx with an IC_{50} of 3-100 μ M (D'hondt et al., 2009; Ma et al., 2009; Ma et al., 2012). Despite their effectiveness as an experimental tool, it is important to note that no single blocker is fully specific to pannexin-1 channels. Thus, employing a combinatorial rule-in/rule-out approach to identifying pannexin-1 activity in this study was the best means of avoiding this potential pitfall.

Table 1. Pharmacological profile of candidate non-selective cation channels

I-V, current-voltage; NMDG⁺, N-methyl-D-glucamin; La³⁺, lanthanum, Ca²⁺, calcium; ECS, extracellular solution; ¹⁰panx1, pannexin-1 inhibitory peptide; TRPM, transient receptor potential melastatin; TRPC, transient receptor potential cation; CRAC, calcium release-activated calcium

	TRPM2	TRPM7	TRPC1/C5	connexin	pannexin	orai/crac
Linear I-V	Yes	No	No	No	Yes	No
NMDG ⁺ permeability	No	No	No	Yes	Yes	No
La ³⁺ block	No	Yes	No	Yes	Yes	Yes
Inhibited by Ca ²⁺ -free ECS	Yes	No	No	Yes	No	No
SKF 96365 block	No	No	Yes	No	No	Yes
Flufenamic acid block	Yes	Yes	n.d.	Yes	No	Yes
Carbenoxolone block	n.d.	n.d.	n.d.	Yes	Yes	n.d.
Probenecid block	n.d.	n.d.	n.d.	No	Yes	n.d.
¹⁰ panx1 block	n.d.	n.d.	n.d.	No	Yes	n.d.
Gp27 block	n.d.	n.d.	n.d.	Yes	No	n.d.

One overlying objective of this project was to provide insight and rationale for exploring a role of pannexin channels in ER stress associated neurodegeneration. Since both pannexin channels and ER stress have individually been shown to contribute to cell death in a variety of cell types, including neurons, this study included experiments to determine whether pannexin channels were activated in response to treatment with A β ₄₂, a known inducer of ER stress.

1.4 Regulation of Ca^{2+} homeostasis by the ER

The ER is a known regulator of intracellular signaling events, most notably intracellular Ca^{2+} homeostasis. When a cell exhausts its capacity to regulate intracellular Ca^{2+} , normal functions are lost and cell death proceeds (Orrenius et al., 2003). Morphologically, the ER extends from dendritic spines, across the cell body, down the axon to the pre-synaptic terminal, which suggests an existing ER contribution to neurotransmitter release and gene transcription among other cellular functions (Verkhatsky and Peterson, 2002). Aside from Ca^{2+} homeostasis, the ER serves many other well-characterized functions including post-translational protein processing as well as the facilitation of protein folding and transport (Burdakov et al., 2005). Correct folding of newly synthesized proteins is aided by several ER chaperone proteins, most commonly binding-immunoglobulin protein (BiP), a heat shock protein family member. Other notable ER chaperones include protein-disulfide-isomerase, calnexin and calreticulin. Once folding is complete, the functional proteins are transported from the ER to the golgi complex (Truettner et al., 2009). Since ER chaperones are Ca^{2+} dependent, their activity is dictated by the intraluminal Ca^{2+} concentration of the ER (Michalak et al., 2002). Therefore, dysregulation of ER Ca^{2+} stores and consequent impairment of ER chaperone function, results in an accumulation of unfolded and misfolded proteins. This disturbance in protein folding characterizes a condition called ER stress (Ryu et al., 2002), a cellular correlate to the progression of neurodegenerative diseases. In fact, the occurrence of cellular stressors that disrupt ER function increases with aging. For example, elevated intracellular Ca^{2+} and reactive oxygen species can alter the balance of the cellular redox

environment to induce ER stress. This trend indicates that the aging brain is more susceptible to ER stress associated neuronal injury (Banhegyi et al., 2008).

1.5 Cellular responses to ER stress

An early cellular response to ER stress is the activation of a pro-survival mechanism that is a network composed of intracellular signaling and transcriptional changes known as the Unfolded Protein Response (UPR). This biochemical cascade involves the coordinated activity of specific enzymes and transcription factors that alter conditions within the ER in an attempt to restore Ca^{2+} and protein homeostasis. The prevailing actions of the UPR are to attenuate the rate at which mRNA is translated, up-regulate the expression of ER resident chaperones and foldases, mediate the expansion of the ER membrane to increase ER compartment space, and trigger ER-associated-degradation to eliminate misfolded proteins (Calfon et al., 2002). Under severe or chronic ER stress, the UPR can stimulate an inflammatory signaling pathway and ultimately apoptosis when pro-survival efforts are exhausted (Harama et al., 2009). In cases of ER stress where homeostasis can not be restored and the signaling pathways of the UPR subverts from pro-survival to pro-apoptotic, fluorescent dyes have been used to monitor the well characterized increase in plasma membrane permeability observed in apoptotic cells (Puthalakath et al., 2007). Given the large pore size and non-selective permeability of pannexin channels, it is reasonable to consider them as the ion channel facilitating this observed response to ER stress.

ER stress markers that are commonly assayed for include the active, dimerized forms of PERK, IRE1 and ATF6 as well as upregulated ER stress responsive genes CHOP and other protein folding chaperones. For the purpose of my thesis, ER stress markers and the signaling pathways of the UPR did not represent the focus of my experimental plan and are therefore not discussed in further detail. Rather, the focus of my study was on the signaling of ER stress to the plasma membrane where a response of ion channel activity could be recorded.

1.6 Store-Operated Ca^{2+} Entry, Stromal Interacting Molecules

A recurring theme in many studies of ER stress is that depletion of ER Ca^{2+} stores is an important trigger for ER stress. This can be enhanced through activation of ryanodine receptors and IP_3 receptors on the ER, or conversely, the impairment of sarcoplasmic-endoplasmic reticulum ATPase (SERCA) function. Experimentally, depletion of ER stores through inhibition of SERCA activity is sufficient to initiate ER stress and cell death (Stutzmann and Mattson, 2011). Typically, ER stress is defined on the basis of UPR induction. However, UPR signaling is unlikely to represent the earliest response of the ER to cellular stressors, which includes depletion of ATP, loss of Ca^{2+} homeostasis and the generation of reactive oxygen species (ROS) (Kohn et al., 1994; Xing et al., 2004). ER chaperones and foldases being Ca^{2+} dependent makes correct protein folding in the ER contingent on luminal Ca^{2+} levels being maintained by steady refilling processes, thus avoiding activation of ER stress pathways.

Stromal-interacting molecules (STIM) proteins, which function as ER resident Ca^{2+} sensors, have recently been identified as important contributors to neuronal cell death post-ischemia where ER stress has been shown to occur and proceed to pro-apoptosis (Berna-Erro et al., 2009). In the mid-1980s, it was proposed that when ER stores are depleted of Ca^{2+} , a refilling process via Ca^{2+} influx from the extracellular environment, involving ER proteins and plasma membrane channels, is utilized (Putney, 1986). This process has been termed store-operated Ca^{2+} entry (SOCE), alternatively referred to as capacitive calcium entry (CCE). Two STIM isoforms (STIM1 and STIM2) have recently been shown to act as Ca^{2+} sensors that relay messages of alterations in luminal Ca^{2+} to the plasma membrane (Liou et al., 2005). Their direct interaction with Ca^{2+} influx channels at the plasma membrane is required for SOCE (Zhang et al., 2006).

1.7 STIM structure and function

The single-pass transmembrane proteins STIM1 and STIM2, originally named GOK and proposed to be involved in tumor suppression and modifications of cell morphology (Sabbioni et al., 1997), have a cytosolic C-terminal, whereas the N-terminal, responsible for Ca^{2+} detection, is in the ER lumen where Ca^{2+} is stored. Functionally, STIM detects decreases in ER-luminal Ca^{2+} through Ca^{2+} binding EF-hand domains on the aforementioned luminal N-terminal. At resting state, defined by a sufficient level of ER-luminal Ca^{2+} , the binding of Ca^{2+} to the intraluminal EF-hand domains of the N-terminal maintains STIM in its resting-dimeric conformation. Also at the N-terminal are dense clusters of sterile- α -motif (SAM) domains that function to stabilize STIM in a dimeric

form when EF-hands are Ca^{2+} bound (Stathopoulos et al., 2006). In the case of depleted Ca^{2+} stores, Ca^{2+} dissociates from the EF-hand domains of STIM, unfolding and destabilizing the EF-SAM clusters. This promotes activation, wherein STIM dimers aggregate into oligomeric STIM complexes (Stathopoulos et al., 2008). Effectively, STIM behaves as a Ca^{2+} sensor and is responsible for relaying the status of ER Ca^{2+} stores to the plasma membrane. Translocation of STIM along the ER membrane to ER-PM junctions enables STIM interaction with Ca^{2+} influx channels expressed at the cell surface to facilitate SOCE.

The two isoforms, STIM1 and STIM2, possess homologous functional domains with the only differences between the two isoforms being slight variations in the amino acid sequences of their N and C termini. Functional consequences of these variations are in their affinities for Ca^{2+} binding, thus affecting each isoform's sensitivity of ER Ca^{2+} detection as well as activation kinetics and contribution to SOCE signaling (Zhou et al., 2009). The higher affinity isoform, STIM1, is regarded as the principal modulator of SOCE because it is rapidly activated under ER Ca^{2+} depletion (Roos et al., 2005), whereas the lower affinity STIM2, despite being more sensitive to ER Ca^{2+} depletion, demonstrates slower activation kinetics and is considered to be responsible for the very fine-tuning of ER Ca^{2+} concentrations (Brandman et al., 2007).

Aside from sensing decreases in ER Ca^{2+} levels, STIM has also been regarded as a general cellular stress sensor because it is also activated by oxidative stress and transient temperature changes (Soboloff et al., 2012). In response to pathological ROS levels, S-

glutathionylation of Cys56 on STIM, adjacent to its EF-SAM domains, causes a dissociation of Ca^{2+} from STIM and subsequent SOCE without ER Ca^{2+} depletion (Hawkins et al., 2010). Additionally, STIM1 has been shown to oligomerize and translocate to ER-PM junctions when cells are heated from 37 to 40°C. Subsequent cooling back to 37°C triggers Ca^{2+} influx, independent of ER Ca^{2+} levels, thus implicating STIM as a sensor to transient temperature change as well (Xiao et al., 2011). So far, members of the ORAI, TRPC and L-type voltage-gated Ca^{2+} channel families have been identified as coupling targets for activated STIM (Feske et al., 2006; Huang et al., 2006; Wang et al., 2010). Given that, the pannexin family of ion channels has been pathophysiologically implicated in various cellular responses associated with ER stress such as hypoxia, ischemia and apoptosis (Dahl and Keane, 2012), STIM is a suitable candidate to consider for coupling ER stress to pannexin channel activation (Fig.1.1). The focus of my thesis is to investigate the relationship between STIM and pannexin channels in the context of ER stress induced pannexin activation.

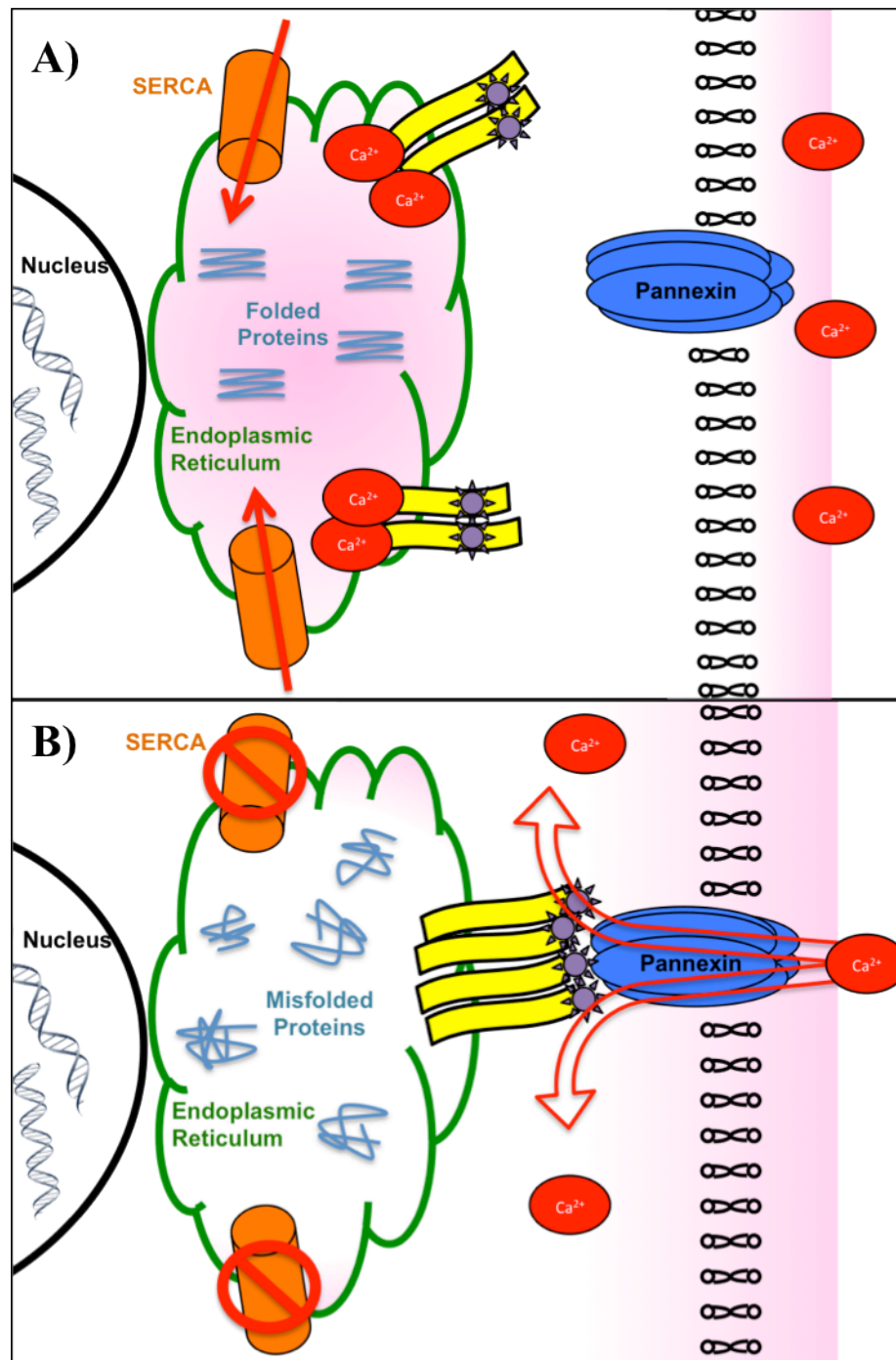


Figure 1.1 Proposed model of ER stress coupled pannexin activity, mediated by activate STIM proteins.

A) At physiological levels of ER- Ca^{2+} , STIM (yellow) remains in resting state as their intraluminal E-F hands bind Ca^{2+} , stabilizing the structure. B) ER- Ca^{2+} depletion and subsequent accumulation of misfolded proteins in the ER, by inhibition of SERCA pumps for example, results in the dissociation of Ca^{2+} from STIM. Active STIM proteins will oligomerize and translocate to ER-PM junctions to interact with plasma membrane ion channels through their CAD domains (purple).

1.8 ER stress in Alzheimer's Disease, beta-amyloid

Neurodegenerative diseases share common pathological features such as the accumulation of misfolded proteins and loss of Ca^{2+} homeostasis. For this reason, ER stress is often studied in the context of neurodegeneration. In particular, ER stress has been well defined in models of Alzheimer's disease (AD), a fatal neurodegenerative disease characterized by progressive dementia and memory loss. Miscoleavage of the amyloid precursor protein in the brain by the gamma-secretase enzyme yields a neurotoxic species, beta-amyloid₁₋₄₂ ($\text{A}\beta_{42}$). A long held view suggests that the deposition of $\text{A}\beta_{42}$ into senile plaques, the hallmark of an Alzheimer's brain at autopsy, is largely responsible for neurodegeneration. However, plaque formation represents the endpoint of an aggregation process which leads to the formation of soluble oligomers, protofibrils and fibrils. Importantly, recent studies suggest that the onset of cognitive decline in patients is better correlated with the occurrence of soluble oligomers rather than plaques (McLean et al., 1999; Mc Donald et al., 2010). Accordingly, a recent focus has been to understand the mechanisms responsible for neurotoxicity induced by soluble oligomers of $\text{A}\beta_{42}$, rather than the formation of plaques, and more specifically, to identify ion channel coupling downstream of $\text{A}\beta_{42}$, a known inducer of ER stress.

Various neurodegenerative diseases, including AD, Parkinson's Disease and Huntington's Disease, are associated with an accumulation of misfolded proteins causing a disruption in ER function, and in turn inducing ER stress (Urano et al., 2000; Imai et al., 2001; Holtz and O'Malley, 2003; Kaneko et al., 2010). In the context of AD at the cellular level, oligomers of $\text{A}\beta_{42}$ disrupt Ca^{2+} homeostasis by altering the function of

ryanodine receptors and IP3 receptors on the ER membrane as well as various calcium permeable channels on the plasma membrane including voltage-gated Ca^{2+} channels, nicotinic acetylcholine receptors and glutamatergic receptors (reviewed by Demuro et al., 2010). thereby causing ER stress and ultimately increasing neuronal vulnerability to apoptosis. Other cellular stresses brought on by oligomers of $\text{A}\beta_{42}$ include increased oxidative stress, disruption of synaptic plasticity, and altered synaptic structure (Townsend et al., 2006; De Felice et al., 2007). Although, the mechanisms responsible for such changes are not well understood recent evidence suggests that $\text{A}\beta_{42}$ may compromise neuronal viability by disrupting the function of the endoplasmic reticulum, leading to ER stress. In AD studies, application of $\text{A}\beta_{42}$ has been shown to increase the expression of ER stress responsive genes (Takahashi et al., 2009) and ER stress markers are detected at the early stages of AD in patients (Hoozemans et al., 2005; Unterberger et al., 2006). Importantly, previous studies have shown that protecting the ER against stress attenuates $\text{A}\beta_{42}$ -mediated neurotoxicity (Lee et al., 2010). In behavioural studies, protection from ER stress has been shown to attenuate memory loss in mouse models of AD (Ricobaraza et al., 2010; Wiley et al., 2011).

1.9 Rationale and hypothesis

Preliminary studies from our lab strongly imply that pannexin channels are activated downstream of ER stress. In these experiments, ion currents were characterized through whole-cell voltage-clamp recordings from pyramidal CA1 hippocampal neurons exposed acutely to commonly used ER stressors. Thapsigargin (Tg) and cyclopiazonic acid

(CPA), although different in structure, are both sarcoplasmic-endoplasmic-reticulum- Ca^{2+} -ATPase (SERCA) inhibitors which were applied to neurons to prevent depleted ER Ca^{2+} stores from being replenished, effectively inducing ER stress (Nguyen et al., 2002). Furthermore, neurons were exposed to tunicamycin (Tn), an inhibitor of N-glycosylation, which decreases the post-translational protein processing typically facilitated by the ER (Shiraishi et al., 2005). The consequent accumulation of unprocessed proteins in the ER initiates ER stress. The observed current was classified as emanating from pannexin channels on the basis of biophysical and pharmacological profiles. Supporting our view was the inhibition of this ER stress-induced current by known pannexin blocking agents such as lanthanum (La^{3+}), carbenoxolone, probenecid and $^{10}\text{panx}$ -inhibitory peptide, as well as its insensitivity to ORAI and TRPC blocker SKF96365 and removal of Ca^{2+} from extracellular solution. What remained to be identified is a mechanism responsible for activating pannexin channels in response to ER stress. One candidate mechanism through which an ER stress signal is relayed to pannexin channels expressed at the cell surface is via coupling with ER-resident STIM proteins, an essential contributor to the refilling of ER Ca^{2+} stores upon depletion.

The primary objective of my thesis was to establish the mechanisms through which ER stress provokes pannexin activation. I hypothesize that pannexin channel activation during ER stress is contingent on the recruitment of STIM proteins.

Section 2

MATERIALS AND METHODS

2.1 Preparation of primary cultured mouse hippocampal neurons

All postnatal hippocampal cell cultures were prepared by technicians from the Jackson/MacDonald lab as previously described (MacDonald et al., 1989). In short, embryonic mouse pups were harvested at embryonic day 17-18, and the hippocampi of each were dissected and isolated. Neurons were dissociated by mechanical trituration using pasteur pipettes and the cells were plated on poly-D-lysine coated petri dishes at a plating density of approximately 50% confluency, approximately 1 million cells per plate. These cultures were incubated in neurobasal culture media, supplemented with B-27 and glucose, at 37°C in 5% carbon dioxide. Media was replenished every 3-4 days. Electrophysiological recordings were performed on cultured hippocampal neurons after 14-21 days in vitro (DIV).

Knockdown of STIM1 and STIM2 was conducted using lentivirus to infect cultured hippocampal neurons with STIM1 shRNA and/or STIM2 shRNA or scrambled shRNA as control. Expression of the shRNA was driven by a u6 promoter. Also expressed on the same transfer vector, pLB, was green fluorescent protein (GFP), which was driven by a CMV promoter. Infection was performed on cultured neurons 3-5 DIV and electrophysiological recordings were performed 14-21 DIV.

2.2 Preparation of pannexin and STIM expressing HEK 293 cells

A cell line of HEK 293 cells stably expressing a tetracycline-inducible pannexin-1 encoding gene “flag-mPannx1”, generated by the Jackson lab, was passaged bi-weekly.

Cells were cultured in 100 mm petri dishes and grown in FBS enriched D-MEM, incubated at 37°C in 5% carbon dioxide. Transient co-transfections of DNA expression plasmids for STIM1 and/or STIM2 as well as GFP were performed on flag-mPannx1 HEK 293 cells using the JetPrime transfection system (Polyplus) prior to electrophysiological recording. To control for the contribution of endogenous STIM expression in some experiments, RNA-silencing vectors targeting STIM1 and STIM2 were also transfected. For experiments involving mutated STIM proteins, DNA expressing an ezrin/radixin/moesin (ERM) domain deleted variant of STIM1 (STIM1 Δ ERM) was used (Fig. 2.1). Briefly, flag-mPannx1 HEK 293 cells were seeded in 6-well plates, 24 hours prior, to yield optimal transfection confluence of 60-80%. Transfection mixes were composed of 200 μ L JetPrime buffer, 2 μ g total DNA, 2.5 μ L JetPrime transfection reagent. 2 μ g of total DNA to be transfected included combinations of DNA vectors encoding GFP, STIM1 (in pCDNA3.1), STIM2 (in pCDNA3.1), shRNA targeting STIM1 expression “pLB(mCherry)-mSTIM1-hp4”, shRNA targeting STIM2 expression gene “pLB(GFP)-mSTIM2-hp4”, a mutated variant STIM1 Δ ERM (in pIRES) and empty vector “pCDNA3.1” (Invitrogen). Transfection mixes were incubated at room temperature for 10 minutes, vortexed for 10 seconds, and added drop wise to flag-mPannx1 HEK 293 cells. Following 4 hours of incubation at 37°C in 5% carbon dioxide, transfection media was replaced with fresh media. Cells were re-seeded on 35 mm petri dishes 24 hours post-transfection at a plating density of 20% for next-day recording or 10% for day-two recordings. Upon plating, 1 μ g/mL doxycycline was added to media to induce pannexin-1 expression.

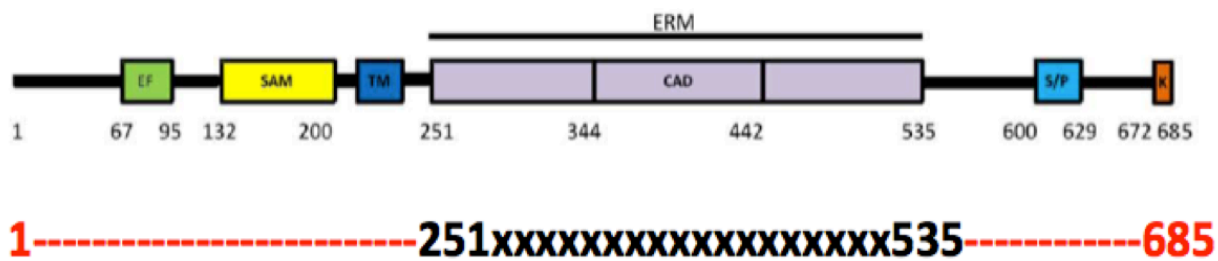


Figure 2.1 Representation of the full length STIM1 protein (above) and the ERM domain deletion in STIM1 Δ ERM variant (below).

2.3 Preparation of oligomeric A β ₄₂ peptides

Solid A β ₄₂ peptides (rPeptide, catalogue no. A-1002-2) stored at -80°C, were equilibrated to room temperature before hexafluoro-2-propanol (HFIP) solvent was added, on ice, to a peptide concentration of 1 mM. After 1 hour of incubation at room temperature with intermittent vortexing to achieve complete dissolution, peptide solutions were placed back on ice for 5-10 minutes. A β ₄₂ solution was aliquoted in 10 μ L samples and HFIP solvent was removed by Speedvac dry down for 10 minutes at 37°C, then placed in -20°C for 2 hours, followed by storage at -80°C leaving only peptide biofilms of A β ₁₋₄₂ monomers. To oligomerize, fresh anhydrous DMSO was added to a concentration of 1 mM A β ₄₂ and vortexed, yielding a final concentration of 100 μ L. Solutions were sonicated for 10 minutes at 37°C then diluted 10-fold in PBS and mixed well. Final incubation at 4°C for 24 hours is when oligomerization occurred yielding soluble oligomers of A β ₄₂, stable at -80°C for up to 2 weeks.

Application of soluble oligomers of A β ₄₂ to primary hippocampal neuron cultures was done 24 hours prior to electrophysiological recording at a final peptide concentration of 1 μ M in media and incubated at 37°C in 5% carbon dioxide.

2.4 Electrophysiology: whole-cell patch-clamp recordings

Pyramidal hippocampal neurons were identified under a microscope by cell shape, with candidate neurons having a triangular shaped cell body, phase-bright contrast, and healthy re-formed non-fractioned processes (Fig. 2.2). For HEK cells, UV light exposure to excite fluorescence in successfully transfected cells was used to visually identify

candidate cells with expression of pannexin-1 and STIM based on GFP and/or Cherry co-expression. Petri dish media was replaced with artificial extracellular solution containing (in mM): 130 NaCl, 25 HEPES buffer, 10 D-Glucose, 10 tetraethylammonium (TEA), 5.4 KCl, 2 CaCl₂ and 1 MgCl₂ adjusted to pH 7.4 with NaOH. The following channel blockers were included in recordings of neurons only (in μ M): 500 4-aminopyridine (4-AP), 0.2 tetrodotoxin (TTX), 50 CdCl₂, 10 bicuculline, 10 cyano-nitroquinoxaline-dione (CNQX), 10 nifedipine. Final osmolarity was adjusted to 305-315 mOsm. Depending on experimental conditions, extracellular solutions would also contain either 3 μ M thapsigargin (Tg) or 100 μ M lanthanum (La³⁺) to evoke pannexin currents or block the channel respectively.

A two-stage pipette puller (Narishige PC-10) was used to make borosilicate glass capillaries under heat to form the patch pipette. The patch pipette was filled with intracellular solution containing (in mM): 130 K-Gluconate, 10 ethylene glycol tetraacetic acid (EGTA), 10 HEPES buffer, 10 KCl, 5 TEA, 4 MgATP, 2 MgCl₂, and 1 CaCl₂ adjusted to pH 7.3 with KOH and osmolarity 295-305 mOsm. The centre of the cell body was approached with a patch electrode of 4-6 M Ω of resistance in solution. Upon approaching the cell, negative pressure was applied to the patch pipette to seal onto the cell membrane. The holding voltage was clamped at -60 mV to mimic the resting membrane potential of a neuron and negative pressure was gently increased until a 1 G Ω seal was achieved. To break through the membrane, while maintaining a tight seal, 0.1 cc of negative pressure was gently re-applied together with a 2 kHz pulse for 0.6 ms. Upon break-through the inner contents of the cell are continuous with the intracellular solution of the patch pipette. Recording protocol, either acute-response (section 2.4.1) or chronic-

response (section 2.4.2), could then be initiated. Traces were recorded using Axoscope and Clampex softwares (Molecular Devices).

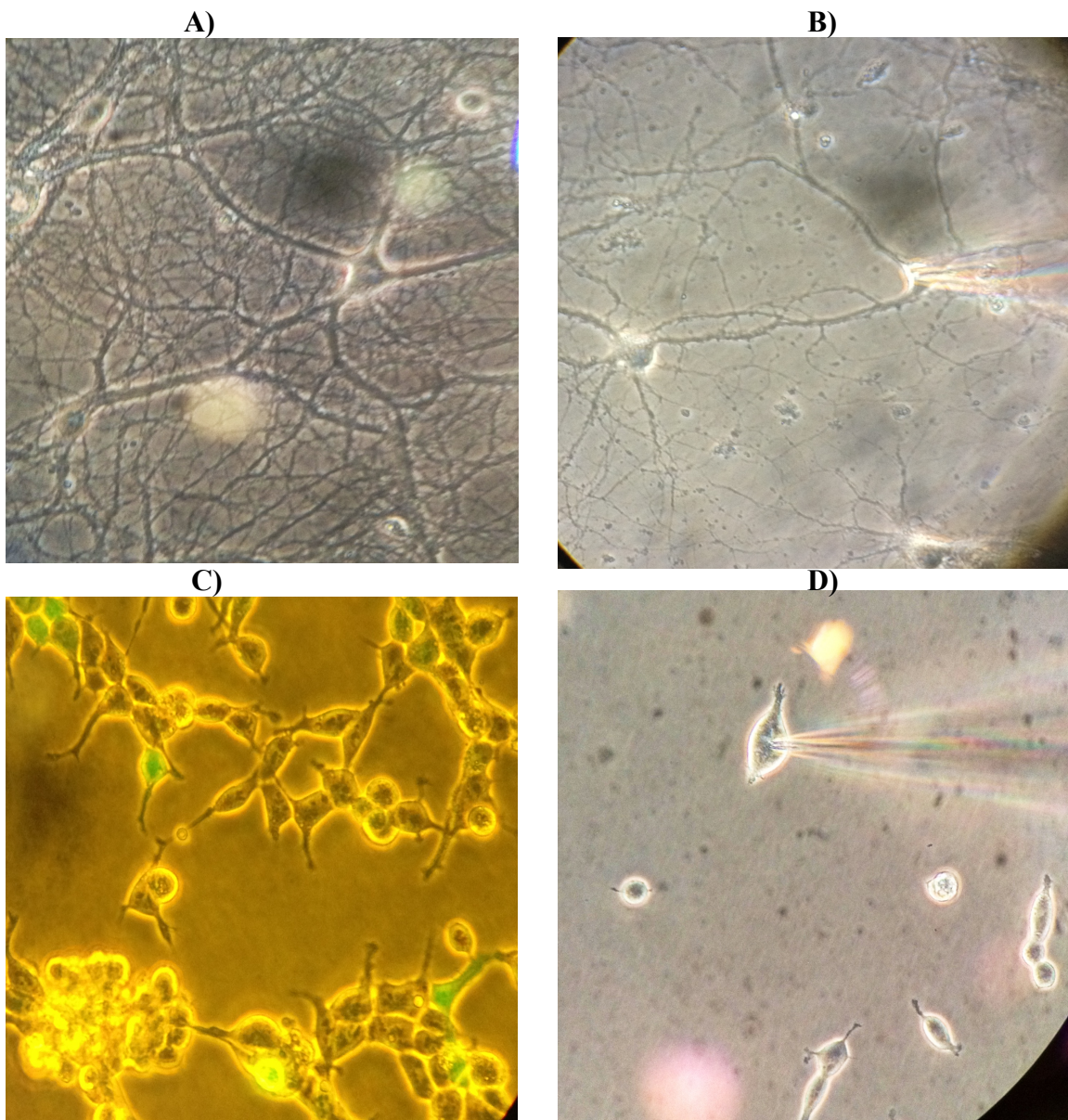


Figure 2.2 **Experimenter's view of cells during patch-clamp recordings.**

A) Pyramidal hippocampal CA1 neuron viewed under phase contrast. B) Pyramidal neuron in whole-cell patched configuration with micropipette. C) Pannexin-1 inducible HEK 293 cells, transfected with GFP tagged STIM. D) Transfected HEK 293 cell in whole-cell patched configuration with micropipette.

2.4.1 Recording acute-response pannexin currents

A shifting multi-barrel perfusion system was loaded with 1) control extracellular solution 2) extracellular solution containing ER stress inducing compound 3) extracellular solution containing pannexin channel blocker (Fig. 2.3). Barrels were positioned to achieve a flow rate of 1 mL/min. Upon break-through of the cell membrane, control barrel was allowed to flow for the duration of a 2-5 minutes stable baseline recording. Initial break-through leak currents of less than -300 pA were considered healthy. Flow was then switched to the ER stress inducing solution barrel for either a 20-minute duration or the development of a characteristic pannexin current. Flow was finally switched to the pannexin blocker solution barrel until either baseline current was re-established or no further decrease in current was made. Traces were analyzed for peak current, residual current after block and/or total charge transfer depending on the objective of the experiment.

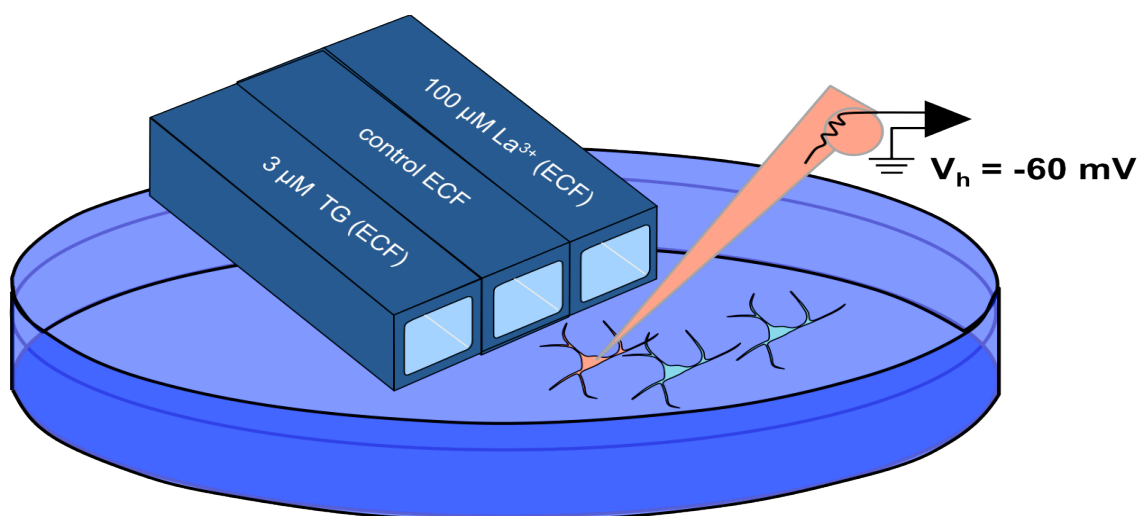


Figure 2.3 Representation of extracellular solution application via a shifting barrel perfusion system in close proximity to the patched neuron

2.4.2 Recording chronic-response pannexin currents

For experiments where acute application of the inducing compound was insufficient in evoking pannexin currents, cells were pre-treated for 24 hours to mimic chronic application. Recording of chronic responses were performed on neurons 24 hours pre-treated with 1 μ M oligomers of A β ₁₋₄₂. Barrel perfusion system was loaded with 1) control extracellular solution 2) extracellular solution containing pannexin channel blocker. Barrels were positioned to achieve a flow rate of 1 mL/min. Recording was started upon gigaseal of patch electrode to cell membrane, prior to break-through. Initial current at the time of break-through was recorded and maintained for 2-5 minutes before flow was switch from control to pannexin blocker solution until either baseline current was re-established or no further decrease in current was made. Traces were analyzed for initial/constitutive current and percentage block of response.

2.5 Statistical analysis

Clampex and Clampfit (Molecular Devices) softwares were used to extract raw data. All data values reported are expressed as \pm standard error. Statistical analysis was conducted using GraphPad Prism 5 (GraphPad Software, CA). Unpaired t-test was used to compare parameters of channel activity between treatment groups. Multiple groups were analyzed by ANOVA with Tukey's post hoc test. For all statistical tests, $P < 0.05$ was considered significant.

2.6 Sources of drugs, reagents and peptides

The sources of drugs, reagents and peptides used in this study are from the following suppliers:

Bishop Canada	KCl, HEPES, EGTA
Enzo Life Sciences	zVAD-FMK
Invitrogen	Thapsigargin, Neurobasal Culture Medium, B-27
Pfizer	Doxycycline
Polyplus	JetPRIME
rPeptide	A β ₁₋₄₂ , A β _{scrambled}
Sigma-Aldrich	NaCl, K-Gluconate, D-Glucose, CaCl ₂ , MgCl ₂ , TTX, TEA, 4-AP, Nifedipine, CdCl ₂ , Mg(ATP) ₂ , La ³⁺ , Probenecid, Carbenoxolone, Dulbecco's Modified Eagle Medium, Fetal Bovine Serum, HFIP, PP2, DMSO

Section 3

RESULTS

3.1 ER stress induces pannexin activation

My first set of experiments was designed to show a relationship between ER stress and pannexin channel activity. Upon successful patching of a healthy candidate cell (described in section 2.4), recording started with 2-3 minutes of exposure with control solution to establish a stable baseline. Thapsigargin, an ER stress inducer by SERCA pump inhibition, at 3 μM in extracellular fluid (ECF) was applied via barrel perfusion system to the patched neuron. In neurons, inward currents indicative of pannexin channel activation developed 10-15 minutes into thapsigargin application. The rate of ECF perfusion was 1 mL/min in all experiments. The evoked inward currents were rapidly blocked by perfusion of 100 μM lanthanum, a reversible blocker of pannexin channels. Reversibility of lanthanum is exhibited by the onset of a large inward current upon washout of lanthanum with control ECF perfusion. Under these conditions, pannexin currents were recovered within seconds (Fig. 3.1A). Thapsigargin evoked pannexin currents were highly reproducible and the sensitivity to lanthanum blockade could be repeated so long as blocker was applied while the cell remained healthy/responsive. Indeed, if the sustained large inward pannexin current were continued for a longer duration, the health of the cells would deteriorate at which time lanthanum could no longer block the much larger currents observed. Therefore, lanthanum ECF was generally applied when developing inward currents either plateaued or reached -2500 to -3000 pA.

In neurons, pannexin currents, identified by their pharmacological sensitivity, were evoked upon treatment with 3 μM thapsigargin ECF (-3010 ± 641 pA, $n=4$), whereas

pannexin currents did not develop under control ECF (-122 ± 29 pA, $n=4$) ($P=0.0041$) (Fig. 3.1C). This serves as a demonstration of ER stress induced pannexin activation.

These results are consistent with recent findings from the Jackson/MacDonald lab illustrating pannexin activation in response to various ER stress inducers, namely thapsigargin and cyclopiazonic acid, both SERCA inhibitors, as well as tunicamycin, an inhibitor of N-glycosylation (Appendix A Fig. S1A). Characterization of pannexin current was previously done in the Jackson/MacDonald lab using a combinatorial approach to rule-in and rule-out candidate non-selective cation channels. Current sensitivity to known pannexin blockers lanthanum, carbenoxolone, probenecid and ¹⁰panx peptide ruled-in pannexin channels. Insensitivity to SKF96365 (ORAI/TRPC channel blocker) and Ca²⁺-free solution (inhibits TRPM/connexin channel activation) further demonstrated the current to be pannexin mediated (Appendix A S1B).

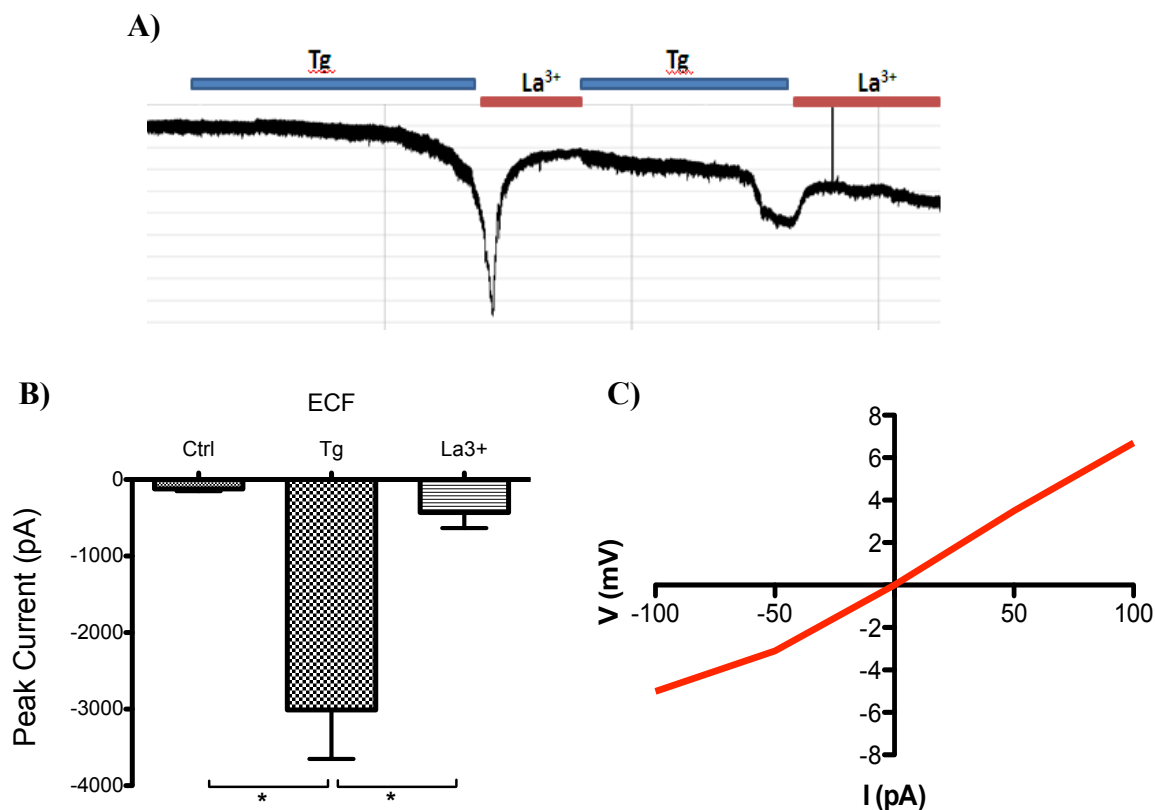


Figure 3.1. Pharmacological ER stress inducers evoke pannexin activation in hippocampal CA1 neurons.

A) Representative trace of whole-cell patch clamp recording from hippocampal CA1 pyramidal neuron. Inward current develops in response to acute treatment of extracellular solution containing 3 μ M thapsigargin (Tg). Current is reversibly blocked by 100 μ M lanthanum (La³⁺) solution. Horizontal bars indicate drug application periods. Voltage was clamped at a holding potential of -60 mV. B) Summary bar graph showing averaged peak currents (pA) under application of control ECF, 3 μ M thapsigargin containing ECF and 100 μ M lanthanum containing ECF in neurons (n=4). C) IV curve, taken at the peaks of currents recorded under Tg were linear, over the voltage range -50 to 100 mV, and reversed at 0 mV, consistent with the IV characteristics of pannexin currents. Values are represented as mean \pm SEM. Statistical analysis: unpaired Student's t-test, P < 0.05.

3.2 A β ₄₂ induced pannexin activation in neurons

Given previous findings that ER stress is associated with various neurodegenerative diseases, I explored the possibility that A β ₄₂ treatment of neurons, known to trigger ER stress (Chefekar et al., 2007), could promote pannexin channel activation. I conducted this experiment in hippocampal CA1 pyramidal neurons, knowing that the hippocampus is important for learning and memory and especially vulnerable to injury in Alzheimer's Disease patients (Troncoso et al., 1996). I first examined the effects of acute A β ₄₂ application during recordings from cultured CA1 neurons. No current was evoked during 60 minutes of constant perfusion of 1 μ M A β ₄₂ in ECF (Fig. 3.2A). This negative finding was not unexpected since A β ₄₂ is not thought to target the ER directly, unlike thapsigargin for instance, and therefore likely requires a more chronic treatment with A β ₄₂. The consequence of chronic A β ₄₂ treatment was therefore examined next in neurons pre-treated with 1 μ M A β ₄₂ for 24 hours. Recording began immediately after forming a seal onto the cell membrane with a resistance greater than 1 gigaohm, but before breaking through the membrane (Fig. 3.2B). 24 hour pre-treatment with A β ₄₂ resulted in large inward current, consistent with known pannexin pharmacology. Pannexin currents were not observed in vehicle and scrambled A β ₄₂ peptide groups ($P < 0.05$) (Fig. 3.2C). These pannexin currents were seen immediately upon breakthrough of the cell membrane, as opposed to developing over minutes as previously seen under thapsigargin treatment, indicative of constitutive pannexin activation in response to chronic (24 hour) A β ₄₂ treatment.

Control experiments were done using vehicle (0.1% DMSO) treatment and a scrambled A β peptide. This ensured that the result was an effect of A β ₄₂, rather than a non-specific

effect of applying a peptide extracellularly. These characteristic pannexin currents were not observed in recordings from either control group (Fig. 3.2C).

To confirm the oligomerization of A β ₄₂, the Jackson/MacDonald lab has tested the final product by western blot (Appendix A Fig. S2).

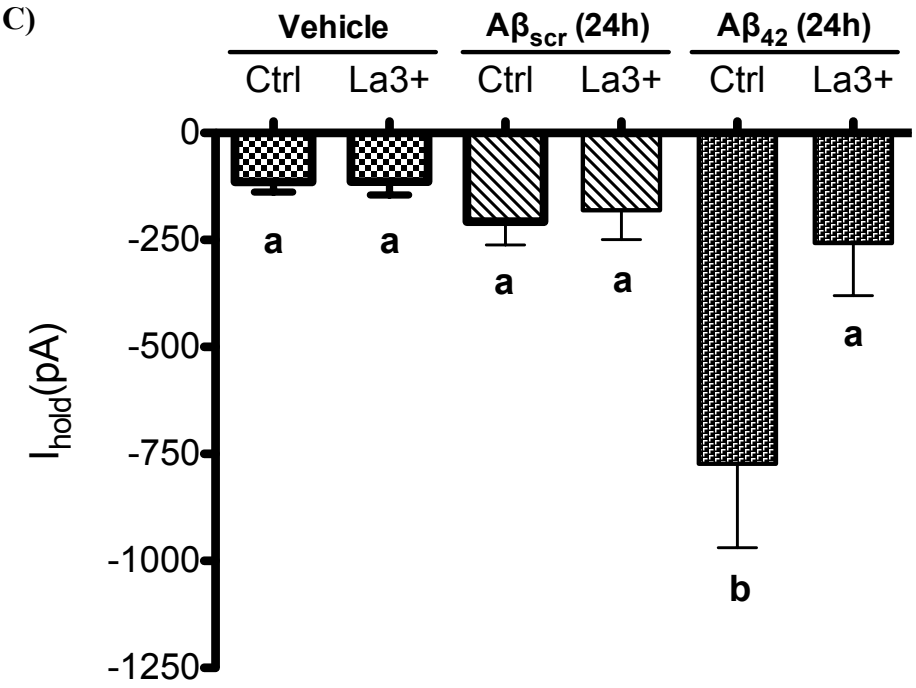
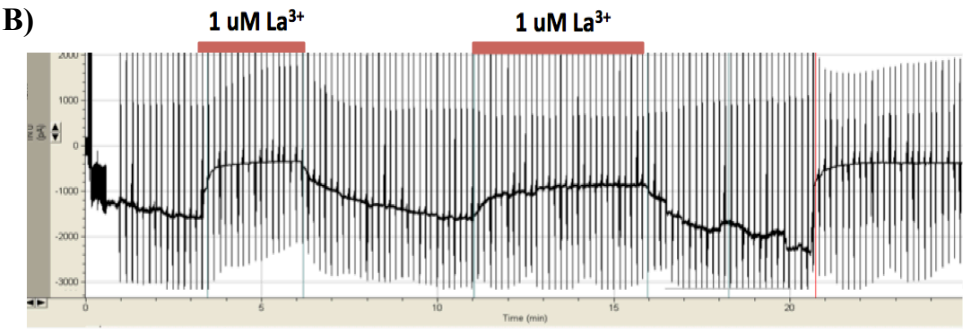
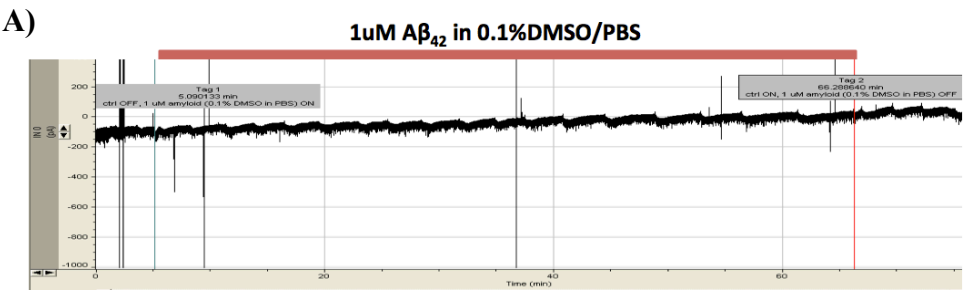


Figure 3.2. Effect of 24 hour extracellular pre-treatment of 1 μ M A β ₄₂ oligomers on pannexin currents in whole-cell voltage-clamp recordings from hippocampal CA1 pyramidal neurons.

A) Lack of current in a recording of acute treatment of 1 μ M A β ₄₂. B) Representative trace of pannexin current from a neuron 24 hours pre-treated with 1 μ M A β ₄₂. C) In the group treated with 1 μ M A β ₄₂ oligomers, constitutive pannexin currents were observed at the point of gaining whole-cell access. These constitutive currents were lanthanum sensitive in the A β ₄₂ group and significantly larger than control groups. Pre-treatment with vehicle (0.1% DMSO) as well as scrambled versions of the A β ₄₂ peptide did not produce pannexin currents. Voltage was clamped at holding potential -60 mV. Values are represented as mean \pm SEM. Statistical analysis: one-way repeated-measures ANOVA with Tukey's post-hoc test. Identical letters (a, b) indicate no significant difference between groups ($P < 0.05$).

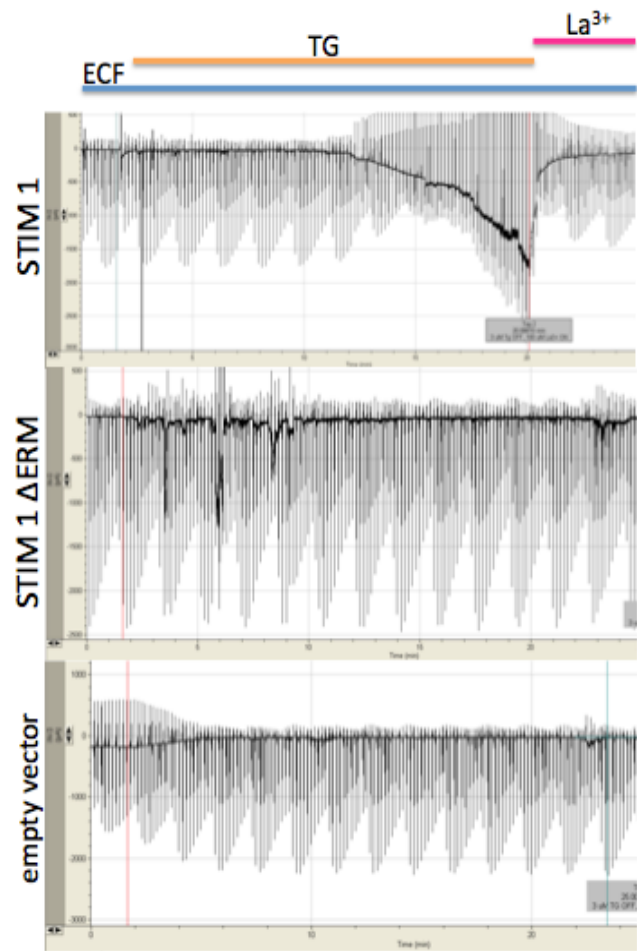
3.3 ER stress induced pannexin-1 activity is modulated by STIM proteins

To identify a molecular mediator in the pathway coupling ER stress to pannexin activation, I investigated the role of the ER stress sensor, STIM, in modulating pannexin currents. I used a mammalian recombinant expression system allowing me to overexpress the proteins of interest, pannexin-1 and STIM. The experimental design was as follows; recordings were performed from HEK 293 cells transiently transfected with pannexin-1 alone (absence of STIM) along with full-length STIM or a mutated variant of STIM. The mutated STIM (STIM1 Δ ERM) possessed a domain deletion such that the ERM domain, which has been shown as the critical domain in ER stress induced ORAI channel activation, was deleted as illustrated in Fig. 2.1 of methods section.

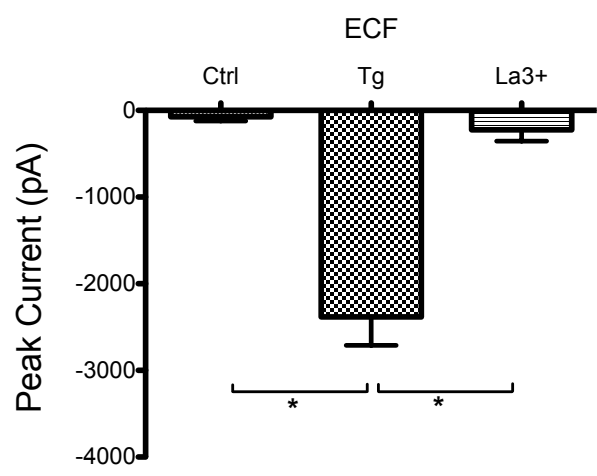
From whole-cell voltage-clamp recordings, application of 3 μ M thapsigargin to HEK cells expressing full-length STIM1 evoked a characteristic pannexin current. Consistent with the known pharmacological sensitivity of pannexin channels and evoked large inward currents during 3 μ M thapsigargin application (-2383 ± 329 pA, n=3). In the absence of thapsigargin, under the perfusion of control ECF, comparable currents were not observed (-73 ± 49 pA, n=3) ($P=0.0023$) (Fig. 3.3B). In contrast, large currents in response to thapsigargin were not observed in the absence of STIM. This is consistent with previous unpublished results from the Jackson/MacDonald lab where thapsigargin did not evoke large pannexin currents in HEK cells that were transfected with pannexin-1 only. Large pannexin currents could only be recorded when co-transfecting either STIM1 or STIM2 along with pannexin-1 (Appendix A Fig. S3). This suggests STIM is required for pannexin activation in response to thapsigargin.

Next, I examined the effects of 3 μ M thapsigargin application to HEK cells expressing a mutated variant of STIM, STIM1 Δ ERM. In contrast to cells expressing full length STIM1, thapsigargin did not evoke pannexin currents in cells expressing the truncated STIM variant (-585 ± 329 pA, n=6) (P=0.12) (Fig. 3.3C). In summary, when comparing the peak currents evoked by thapsigargin between STIM1 and STIM1 Δ ERM groups, the current is significantly larger in the STIM1 group (p=0.0113) (Fig. 3.3D).

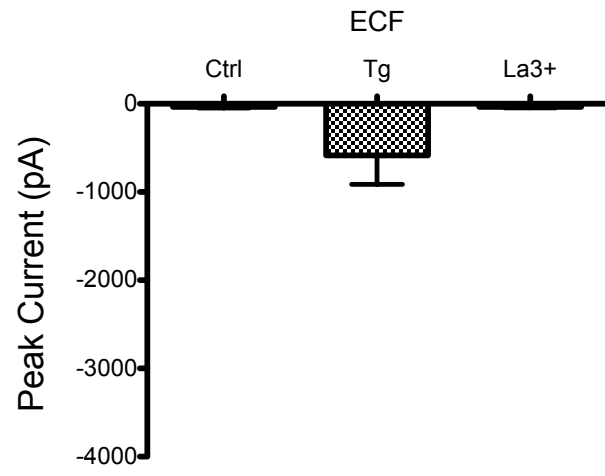
A)



B) STIM1



C) STIM1ΔERM



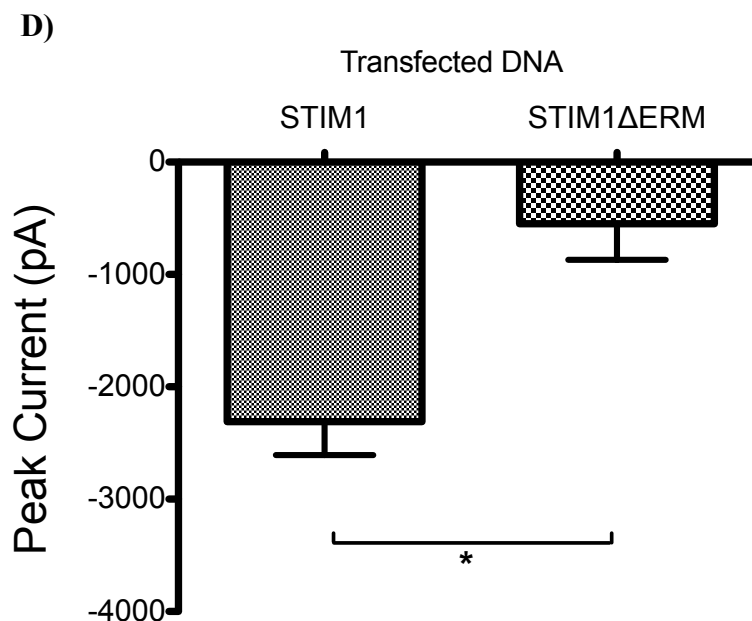


Figure 3.3. Effect of ERM-domain deletion from full-length STIM on ER stress induced pannexin-1 currents in whole-cell voltage-clamp recordings from HEK 293 cells transiently transfected with pannexin-1 and either full-length or mutated STIM1. **A)** Representative traces of whole-cell patch clamp recordings from transiently transfected HEK 293 cells overexpressing pannexin-1 and either full length STIM1, mutated STIM1 with deletion of the ERM domain or empty vector ie. no expression of STIM1. Horizontal bars indicate drug application periods. Summary bar graph showing peak current (pA) under application of control ECF, 3 μ M thapsigargin-containing ECF and 100 μ M lanthanum-containing ECF with overexpression of **B)** full length STIM1 (n=3) and **C)** mutated STIM1 (n=6). **D)** Comparison of peak current between full length STIM1 and mutated STIM1 during thapsigargin treatment. Voltage was clamped at holding potential -60 mV. Values are presented as mean \pm SEM. Statistical analysis: unpaired Student's t-test, $P < 0.05$.

3.4 ER stress evoked pannexin current is not STIM-dependent in neurons

After observing ER stress induced pannexin activation in both neurons and HEK cells, followed by evidence of this response being STIM-dependent in HEK cells, the next step was to determine if pannexin activation in neurons also required STIM. This would establish a mechanism for coupling ER stress to pannexin activation in neurons and provide a candidate mechanism for the aforementioned A β ₄₂ induced pannexin activation. To investigate STIM-dependent pannexin activation in neurons, I recorded ER stress induced pannexin currents from hippocampal CA1 pyramidal neurons where expression of STIM1 and STIM2 was knocked down after 14-16 days of lentiviral transduction with shRNA sequences targeting STIM1 and STIM2. Fluorescent tags, GFP for STIM1 and mCherry for STIM2, were expressed on the same vector as the shRNA sequences. This allowed for visual identification and selection of infected neurons for whole-cell recordings.

To evaluate the effectiveness of the shRNA in knocking down expression of the proteins of interest, the Jackson/MacDonald lab performed western blots assaying for levels of STIM1 and STIM2 using whole-cell protein extracts 14-16 days post-infection with shRNA. Levels of STIM1 and STIM2 were quantified densitometrically and normalized to β -actin. Results show that the expression of STIM1 and STIM2 was effectively knocked down by 70-90% from control levels by their respective shRNA target sequences (Appendix A S4).

When comparing peak currents, evoked by 3 μ M thapsigargin, between the STIM knockdown group (-1685 ± 292 pA, n=5) and the control group (-2016 ± 200 pA, n=6), no significant difference was observed ($P=0.3620$) (Fig. 3.4A). In the same recordings,

residual current remaining after 100 μ M lanthanum was also analyzed. STIM knockdown group (-487 ± 192 pA, n=5) and control group (-560 ± 167 pA, n=6) showed no significant difference ($P=0.7749$) (Fig. 3.4C). Another parameter analyzed was total charge transferred across the membrane, for the duration of the thapsigargin application, as measured by the area under the curve (i.e. time integral of membrane current). No significant difference was observed between STIM knockdown (-216 ± 46 pA, n=5) and control group (-237 ± 82 pA, n=6) for total charge transfer ($P=0.8336$) (Fig. 3.4E). Since it is not certain that the knockdown of STIM expression was 100% effective, I needed to address the possibility that 3 μ M thapsigargin may be sufficient to evoke an ER stress response mediated by the residual STIM protein, post-knockdown. Therefore, these recordings were repeated with 1 μ M thapsigargin application instead of 3 μ M. However, large inward currents were nevertheless observed in response to this lower concentration of Tg.

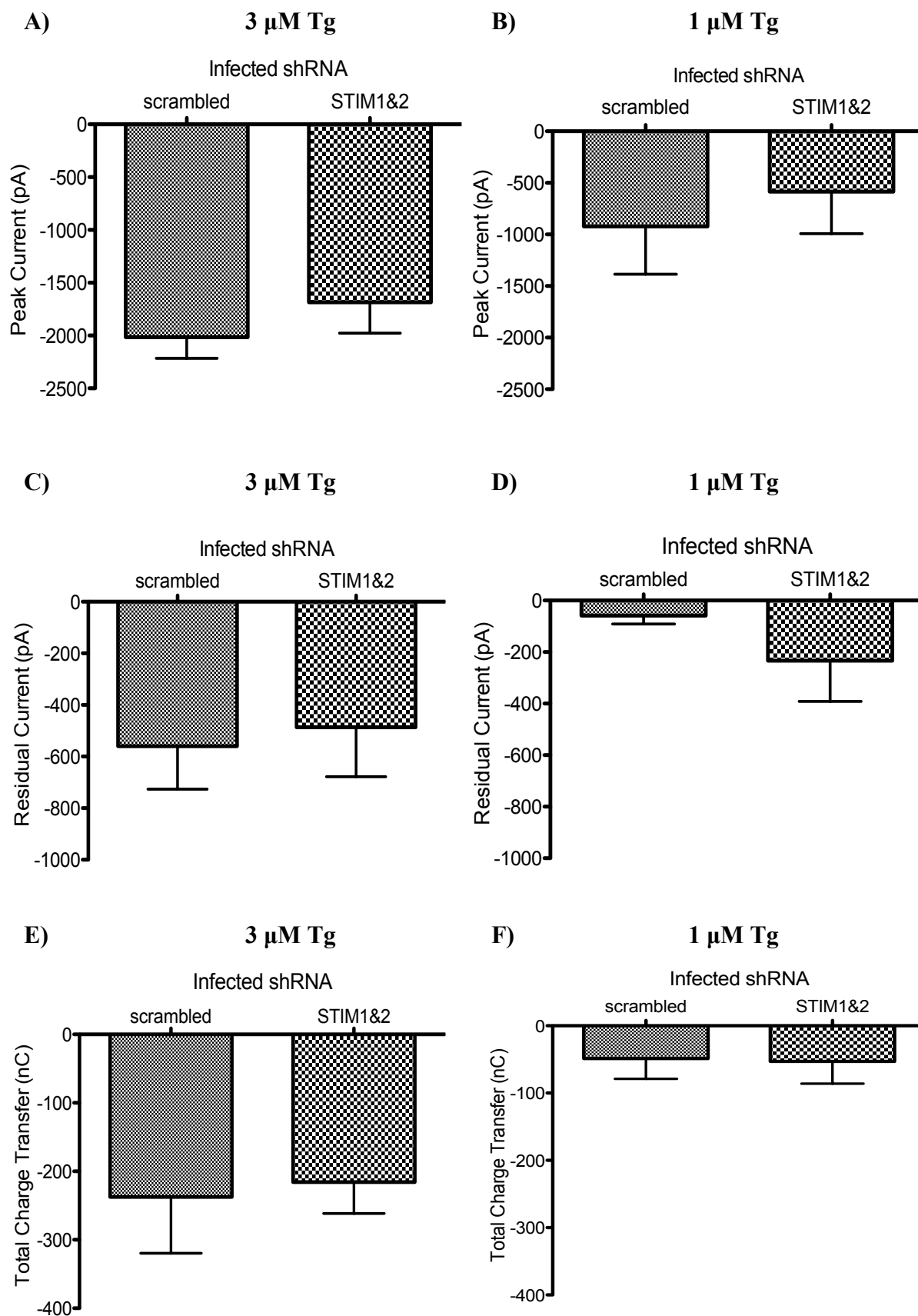


Figure 3.4. Effect of knockdown of STIM1 & 2, via RNAi, on ER stress pannexin currents in whole-cell patch-clamp recordings from hippocampal CA1 pyramidal neurons.

A) Peak pannexin current was not altered in response to 3 μ M thapsigargin **B)** nor in response to 1 μ M thapsigargin. **C)** Residual pannexin current after blockade with 100 μ M lanthanum was not altered in response to 3 μ M thapsigargin **D)** nor in response to 1 μ M thapsigargin. **E)** Total charge transfer, as determined by area under curve of current amplitude over time recording, was not altered in response to 3 μ M thapsigargin **F)** nor in response to 1 μ M thapsigargin. Voltage was clamped at holding potential -60 mV. Values are represented as mean \pm SEM (STIM1&2 KD, n=5; scrambled, n=6). . Statistical analysis: unpaired Student's t-test, $P < 0.05$.

3.5 ER stress evoked pannexin current is not Src-kinase dependent in neurons

My findings suggest that STIM is not required to facilitate ER stress induced pannexin channel activation in cultured neurons. I therefore examined alternative candidate mechanisms for promoting pannexin activation in neurons. In light of recently published findings in which Src-family kinases were shown to mediate neuronal pannexin channel activation induced by hypoxia (Weilinger et al., 2012), I decided to investigate the role of Src-family kinases in ER stress induced pannexin activation. In the study from the Thompson lab, inhibition of Src-family kinases was found to reduce pannexin current size and cumulative charge transferred across the membrane during pannexin activation. My experiment was designed to assess the requirement of Src by examining whether the specific inhibition of Src can prevent the activation of pannexin channels in response to ER stress inducer thapsigargin. To inhibit Src-family kinases, I used the drug PP2 at a concentration of 10 μ M in ECF, which was the effective concentration used by the Thompson lab.

Unlike the results of the Thompson lab, my recordings do not show a reduction in peak thapsigargin-evoked pannexin currents between Src-inhibitor treated (-1883 ± 188 pA, $n=6$) and control recordings (-2352 ± 225 pA, $n=5$) ($P=0.1399$) (Fig. 3.5A). When comparing residual current remaining after 100 μ M lanthanum was applied, again there is no difference between neurons treated with 10 μ M PP2 (-986 ± 117 pA, $n=6$) and control recordings (-823 ± 185 pA, $n=5$) ($P=0.4588$) (Fig. 3.5B). Finally, comparison of total charge transfer, measured by area under the curve in these same recordings, was also the same between 10 μ M PP2 treated (-149 ± 112 pA, $n=6$) and control groups (-237 ± 60 pA, $n=5$) ($P=0.5383$) (Fig. 3.5C). The results of this experiment suggest the coupling of

ER stress to pannexin channel activation in neurons is independent of phosphorylation by Src-family kinases.

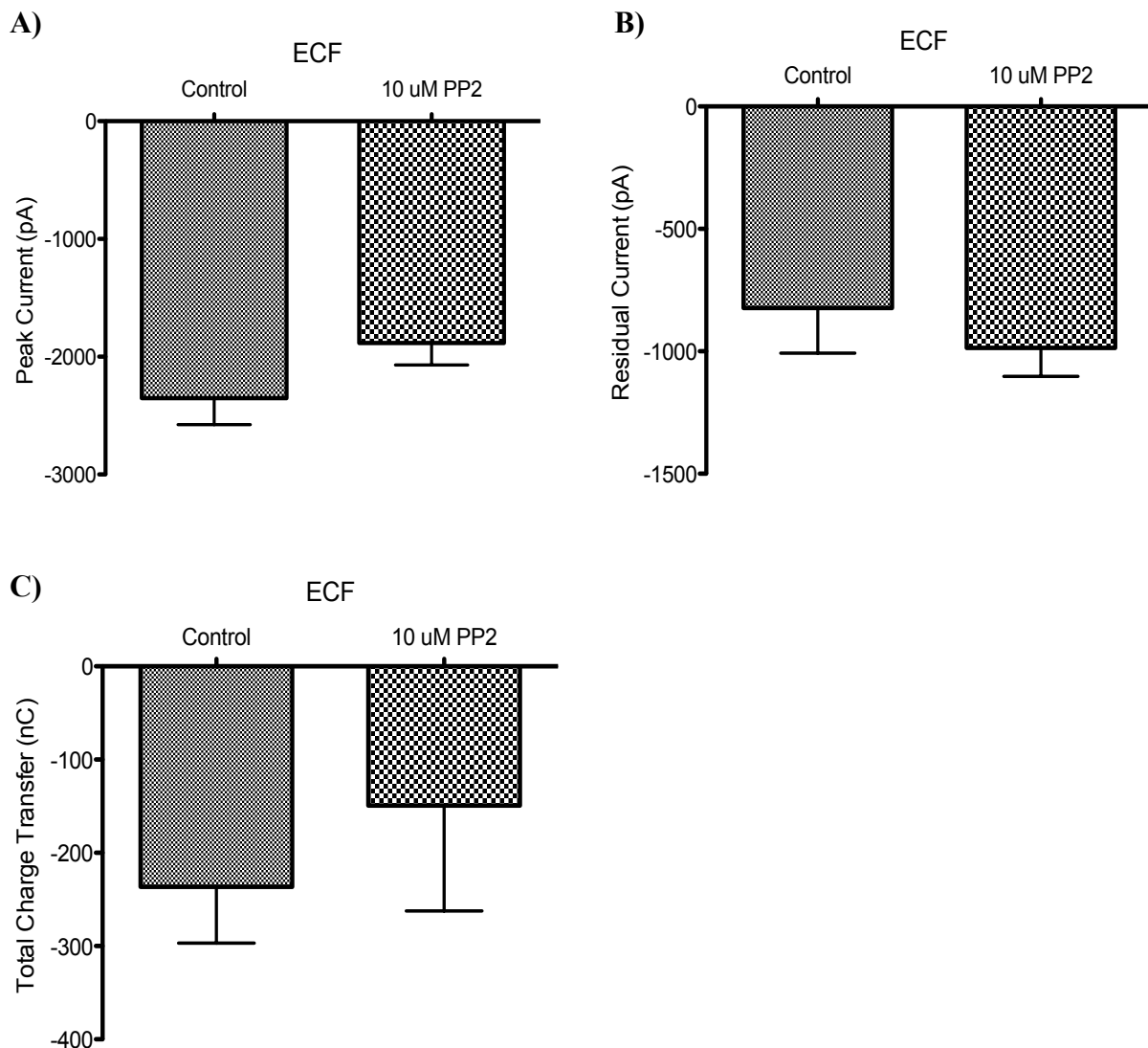


Figure 3.5. Effect of Src-family kinase inhibition on ER stress induced pannexin currents in whole-cell patch-clamp recordings from hippocampal CA1 pyramidal neurons.

Recordings were performed in the presence of Src-family kinase inhibitor PP2 at 10 μM in ECF. **A)** Peak pannexin current during 3 μM thapsigargin application was not altered between Src-inhibitor and control groups. **B)** Residual pannexin current after blockade with 100 μM lanthanum application was not altered between Src-inhibitor and control groups. **C)** Total charge transfer, as determined by area under curve of current amplitude over time recording, was not altered between Src-inhibitor and control groups. Voltage was clamped at holding potential -60 mV. Values are represented as mean \pm SEM (10 μM PP2, n=6; control, n=5). Statistical analysis: unpaired Student's t-test, $P < 0.05$.

3.6 ER stress evoked pannexin current is not caspase-dependent in neurons

After ruling out STIM and Src-family kinases as mediators of ER stress induced pannexin activation in neurons, I examined whether yet another candidate mechanism was responsible, namely caspase-mediated cleavage of the pannexin channel. Indeed, a previously identified pathway in pannexin activation during apoptosis by the Ravichandran lab suggested that cleavage of the intracellular C-terminus of pannexin channels by caspase 3 or caspase 7 was necessary to open the channel pore during pannexin activation (Chekeni, 2010). Their use of intracellular 20 μ M zVAD-FMK, a commercially available caspase inhibitor, was effective in preventing pannexin current from developing under apoptosis inducing conditions in jurkat cells expressing pannexin-1 channels. My experiment was designed to observe the effects of inhibiting caspase 3 and caspase 7 in whole-cell recordings of ER stress induced pannexin currents in neurons. To inhibit caspases 3 and 7, I used the same compound zVAD-FMK at a concentration of 20 μ M in my intracellular fluid (ICF) solution, which was the effective concentration used by the Ravichandran lab.

In these recordings, application of 3 μ M thapsigargin reliably evoked pannexin currents as seen in control neurons. Current size and sensitivity were identical to control recordings. Additional recordings in neurons confirmed that 20 μ M zVAD-FMK was ineffective in preventing pannexin activation in response to thapsigargin. This suggests that caspase cleavage is not required for ER stress induced pannexin activation.

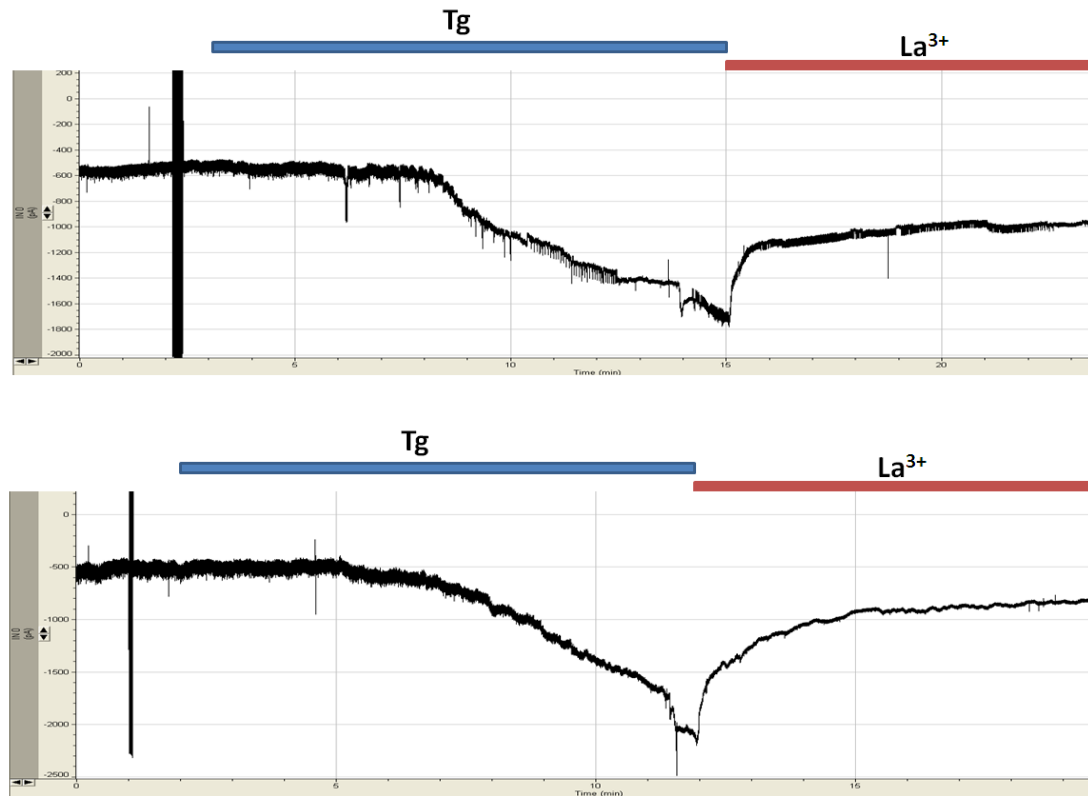


Figure 3.6. Effect of caspase inhibition on ER stress induced pannexin currents in whole-cell patch-clamp recordings from hippocampal CA1 pyramidal neurons.

Both recordings were performed in the presence of caspase inhibitor, zVAD-FMK at 20 μ M in ICF. Traces of recordings from preliminary trials demonstrating pannexin current was not altered by caspase inhibitor in response to 3 μ M thapsigargin. Pannexin currents were identified by sensitivity to pharmacological pannexin blockers. Pannexin current block by application of 100 μ M lanthanum was not altered by the presence of intracellular caspase inhibitor. Voltage was clamped at holding potential -60 mV.

Section 4

DISCUSSION

4.1 Summary of key findings

In this project, I investigated potential mechanisms underlying pannexin channel activation in response to ER stress. Specifically, I explored the involvement of STIM proteins in mediating this response and aimed to identify a critical domain that was required to facilitate an interaction. I confirmed previous unpublished results from our lab wherein ER stress inducers activated pannexin currents in neurons, in accordance with the pharmacological profile of pannexin-1 channels. I then identified this activation to be contingent on the recruitment of STIM in the HEK cell expression system. Furthermore, I identified the ERM domain of STIM as a critical domain in activating pannexin as suggested by the lack of pannexin current when overexpressing STIM molecules with deleted ERM domains in HEK cells. In neurons, however, knockdown of STIM proteins was not sufficient in abolishing ER stress induced pannexin currents as noted by amplitude peak current, residual current as well as total charge transfer. Upon exploring alternative mediators of ER stress induced pannexin activation, comparable pannexin currents were evoked independent of Src-family kinases and cleavage by caspases. To summarize the key results, pannexin activation in response to ER stress has been established, however, a molecular mechanism underlying this activation has been demonstrated in HEK cells but not yet in neurons.

4.2 Pannexin channel activation in response to ER stress

Using whole-cell voltage-clamp recordings of pannexin currents from cultured pyramidal CA1 neurons of mouse hippocampus, ER stress was induced by acute treatment with

commonly used ER stressors. Our lab has used 3 different inducers of ER stress, namely, SERCA pump inhibitors thapsigargin and cyclopiazonic acid as well as N-glycosylation inhibitor tunicamycin. Each of these agents has been shown to reliably induce ER stress in a number of different systems (Tobmal et al., 2000; Lehrman, 2001; Pirot et al., 2006). These agents all act upon the ER and are thought to provoke the accumulation of misfolded proteins in the ER. This occurs either as a consequence of disrupting ER Ca^{2+} homeostasis (thapsigargin and cyclopiazonic acid) or by interfering with glycosylation (tunicamycin), both of which are necessary for regulated protein folding in the ER. The consequent accumulation of unprocessed proteins, brought on by the disruption in Ca^{2+} homeostasis and misfolding of proteins within the ER, initiates ER stress (Michalek et al., 2002). These three pharmacological ER stress inducers have been shown by our lab to reliably produce pannexin channel activation (Appendix A S1A). In a first series of experiments, I have confirmed the activation of pannexin channels by the ER stressor, thapsigargin. I was able to reproduce this result in cultured pyramidal neurons from the CA1 region of mouse hippocampus. Acute extracellular application of 3 μM thapsigargin while recording from whole-cell patch-clamped cells, consistently evoked a 2000-3000 pA current that could be reversibly blocked with the application of 100 μM lanthanum (Figure 3.1).

Stemming from the lack of a specific blocker for pannexin channels, a common criticism towards pannexin channel identification is in the certainty that the recorded current is in fact pannexin. Accordingly, our lab has extended this result over the known pharmacological profile of pannexin channels to positively identify the currents generated

as being pannexin mediated. Thus, we have shown these currents to be sensitive to lanthanum, carbenoxolone, probenecid, ¹⁰panx peptide, as well as insensitive to the ORAI and TRPC channel blocking compound, SKF96365; and also the connexin and TRPM blocking condition, calcium-free extracellular recording solution. The resulting pharmacological profile uniquely identifies pannexins channels as being responsible for ER stress activated currents.

4.3 ER stress-pannexin coupling modelled in neurodegenerative disease

Although pharmacological inducers provide a reliable experimental means of modeling ER stress, the next step was to demonstrate the relevance of ER stress induced pannexin activation in neurological disease. My objective was to extend these findings by determining whether pannexin activation is observed in neurological disease where ER stress is known to occur. ER stress has been demonstrated in response to exposure of cultured neurons to A β ₄₂. In terms of the molecular players involved, the three branches of the UPR start with activating-transcription-factor-6 (ATF6), protein-kinase-RNA-like-ER-kinase (PERK), and inositol-requiring-enzyme-1 (IRE1). In the absence of ER stress, BiP, also an ER resident protein, remains bound to PERK, IRE1, and ATF6, suppressing their signaling activity. However, when the protein folding capacity of the ER is exhausted and unisfolded proteins accumulate, BiP dissociates from its binding partners and binds to misfolded proteins, initiating the UPR (Schroder and Kaufman, 2005). These three active constituents (ATF6, PERK, IRE1) and BiP are typically screened for as markers of ER stress.

Previous studies have shown increases in UPR markers such as PERK, IRE1, ATF6 and BiP in response to A β ₄₂ application (Katayama et al., 2003; Ferreira et al., 2006; Seyb et al., 2006). The aforementioned findings of my earlier experiments showing ER stress coupled pannexin activation provided a rationale for me to investigate whether ER stress by A β ₄₂ can promote pannexin channel activation. In these experiments, I showed constitutive activation of pannexin channels in response to 24 hours of pre-treatment with A β ₄₂. Currents recorded upon initial breakthrough during whole-cell voltage-clamp recordings were significantly larger than those in the vehicle treated group (Figure 3.2C). Recorded currents were identified as pannexin currents based on their sensitivity to 100 μ M lanthanum blockade as well as their lack of sensitivity to other channel blockers contained in recording solutions (TEA, TTX, CNQX, bicuculline, 4-AP, nifedipine). This finding is the first to demonstrate pannexin channels activation in a model of Alzheimer's Disease.

Thus far, I have not defined a role for pannexin channels in the context of neurodegeneration. Given the previous implications of pannexin channels mediating cell death and inflammation, it is conceivable that pannexin channels could be functioning in a similar role in Alzheimer's Disease. Whether or not pannexin channels contribute to A β ₄₂ induced neurotoxicity remains to be determined. Therefore, a follow-up experiment to this study would be to examine if the absence of pannexin channels, either by knockdown or knockout, reduces neuronal cell death in this model. This can be assessed using either an MTT assay to measure cell viability based on metabolic activity, or using live/dead staining to assay for membrane integrity.

4.4 Molecular basis for pannexin activation during ER stress

The primary objective of my study was to establish the mechanisms through which ER stress provokes pannexin activation. I hypothesized that pannexin channel activation during ER stress is contingent on recruitment of STIM proteins. My electrophysiological data in transfected HEK 293 cells was strongly in favour of my hypothesis. Only cells transfected with full length STIM and inducibly expressed pannexin-1 had ER stress evoked currents that were pharmacologically identified as pannexin currents (Fig. 3.2). In contrast, the cells transfected with an empty vector or just GFP, for visual confirmation of transfection efficiency, were not able to generate a pannexin current in response to pharmacological ER stress induction. This group portrayed the absence of STIM and made it evident that ER stress induced pannexin activation was contingent on the recruitment of STIM. In these experiments, it was expected that depletion of ER Ca^{2+} allowed Ca^{2+} to dissociate from the intraluminal N-terminal E-F binding hands of STIM, normally maintained as homodimers at rest when the ER is replenished. The dissociation of Ca^{2+} provokes a conformational change that releases the auto-inhibitory domain of STIM from binding to itself. This activates STIM proteins and allows them to oligomerize and translocate as a cluster to regions of the ER that are in close proximity to the plasma membrane. Within these ER-plasma membrane junctions we propose that STIM proteins physically interact with plasma membrane bound pannexin channels to promote their activation. Precedence for the interaction of STIM with plasma membrane channels has been established in the context of ORAI activation during store-operated Ca^{2+} entry, as modeled in Figure 4.1.

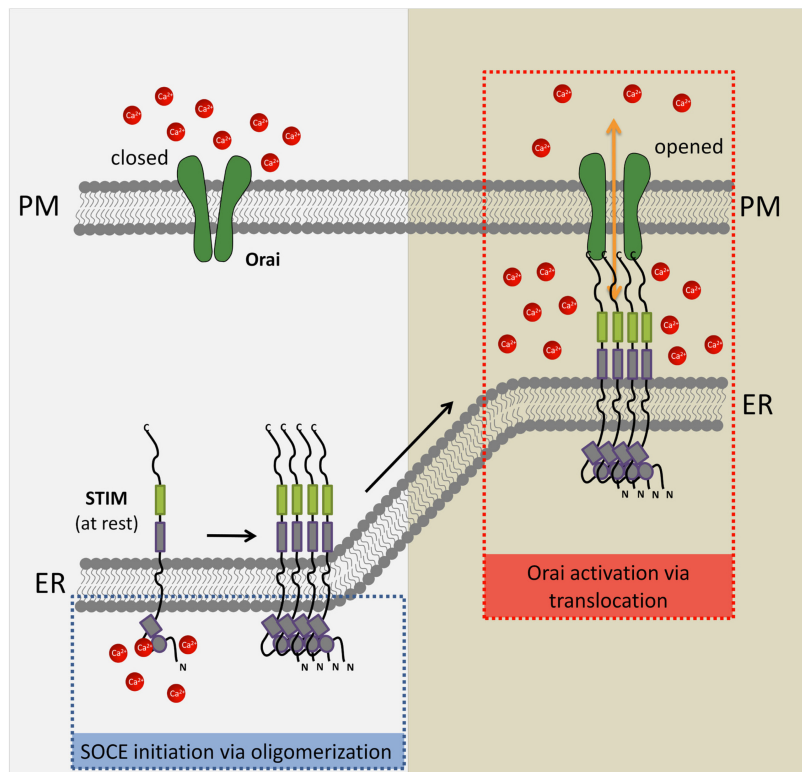


Figure 4.1 Schematic model of STIM-ORAI interaction during store-operated Ca^{2+} entry

Upon ER- Ca^{2+} store depletion, STIM oligomerizes and translocates towards ER-PM junctions where it interacts with and activates Ca^{2+} permeable ORAI channels to replenish ER- Ca^{2+} stores. (Modified from Roos et al., 2005)

In this context, recent evidence suggested STIM proteins not only participate in store-operated Ca^{2+} entry to regulate ER Ca^{2+} refilling but may also function more broadly as a sensor for ER stress (Soboloff et al., 2011). Independent of ER Ca^{2+} depletion, STIM activated by reactive oxygen species (ROS) couples to ORAI channels suggesting STIM also functions as a sensor of oxidative stress (Hawkins et al., 2010). Additionally, cellular stress from transient temperature variation can also induce STIM-ORAI coupling independent of ER Ca^{2+} depletion (Xiao et al., 2011). Upon further review of the literature describing ORAI channel activation during store-operated Ca^{2+} entry, it has been reported that the Ca^{2+} -channel activating domain (CAD) region of STIM1, residues

342-348, is sufficient to constitutively activate ORAI channels (Park et al., 2009). In its resting conformation, the CAD region of STIM1 is auto-inhibited by intramolecular electrostatic interactions. Activation of STIM triggers conformational changes exposing the acidic residues of the CAD region to interact with the basic sequences on ORAI (Wang et al., 2010). This information led me to investigate whether the CAD region of STIM was crucial in activating pannexin channels during ER stress, analogous to its role in activating ORAI channels during store-operated Ca^{2+} entry. Experimentally, I needed a system where mutated STIM proteins, with deleted CAD regions, could be expressed. Therefore, instead of recording in cultured neurons where I could not readily control STIM expression levels, I again used HEK cells expressing pannexin-1 (doxycycline-inducible) where I could transiently transfect DNA encoding for mutated STIM proteins. First, I demonstrated that in the absence of transient expression of STIM, thapsigargin-evoked pannexin-1 currents are absent in these cells (Figure 3.3A), a result I reproduced from previous Jackson/MacDonald lab data (Appendix A S3). One control experiment that remains to be completed is the recording of ER stress induced current in HEK cells expressing STIM but not expressing pannexin-1, an experiment where we do not expect current to be evoked.

To advance that finding, I recorded panx1 currents in response to 3 μM thapsigargin in HEK cells expressing pannexin-1 (induced with doxycycline) with transient transfection of mutated STIM1 whereby the ERM domain of STIM1, which contains the CAD region, was deleted from the full-length protein ($\text{STIM1}_{\Delta\text{ERM}}$). The lack of current that developed in the $\text{STIM1}_{\Delta\text{ERM}}$ group compared to the full length STIM group provided

evidence that the ERM domain is a critical domain in mediating the coupling of ER stress to pannexin activation (Figure 3.3D). This experiment reinforces the notion that a physical interaction may exist between active oligomeric STIM complexes and pannexin channels on the plasma membrane, analogous to the well-established interaction between active oligomeric STIM complexes and ORAI channels on the plasma membrane. Whether such an interaction between STIM and pannexin is direct or indirect, via a macromolecular complex, is yet to be determined and provides a rationale for future co-immunoprecipitation experiments on STIM-pannexin as well as CAD-pannexin in a model of ER stress.

4.5 Contribution of STIM proteins to pannexin channel activation in neurons

After demonstrating ER stress induced pannexin activation pharmacologically and also with A β ₄₂, I sought to identify the underlying mechanisms which couple ER stress to pannexin activation in neurons. My own results suggest that STIM, and more specifically its ERM domain, is required for pannexin activation in HEK cells in response to treatment with ER stressors. This led me to predict that a knock down of STIM proteins in neurons would abolish ER stress induced pannexin currents. To test this idea, I recorded in neurons which had been lentiviral infected with DNA encoding for short RNA hairpins targeting STIM transcripts (shRNA_{STIM1} and shRNA_{STIM2}) to knockdown expression of STIM proteins. Separate biochemical experiments from sister cultures infected in parallel confirmed the reduction of STIM expression (Appendix A S4). Opposing the prediction, thapsigargin-induced pannexin currents were evoked in STIM

knockdown neurons with no difference observed in peak current, residual current after 100 μ M lanthanum block or total charge transferred across the plasma membrane between the knockdown and the scrambled shRNA groups (Figure 3.4 A, C, E). It was conceivable that 3 μ M thapsigargin was a high enough concentration to evoke an ER stress response mediated by only the residual STIM (10-30% of control levels) left behind after shRNA knockdown. For this reason, I repeated the experiment using a lower dose, 1 μ M thapsigargin. However, pannexin currents of similar size and progression were evoked under the low dose as well, indicating the response was not dose-dependent at thapsigargin concentrations greater than 1 μ M (Figure 3.4 B, D, F).

When interpreting the functional consequence of this result, at face value the result of my STIM-KD experiment in neurons (Figure 3.4) suggests that natively expressed STIM does not contribute to pannexin channels activation in response to Thapsigargin treatment of cultured hippocampal neurons. One pitfall of the approach utilized is that recordings may have been made from neurons in which less than complete knockdown of STIM has been achieved. To maximize the likelihood of recording only from certain successfully infected neurons, each lenti-transfer vector, used to express shRNA sequences, also allowed expression of different fluorescent marker (GFP and RFP). shRNA_{STIM1} was co-expressed with GFP and shRNA_{STIM2} was co-expressed with mCherry. By selecting only neurons that were GFP-positive as well as mCherry-positive, I could confirm that the neurons I recorded from did express both shRNA variants. Using this criteria I observed an infection efficiency of 90-100% in cultured neurons. Our western blot data suggests substantial reduction (~70-90%) in STIM1&2 after lentiviral shRNA infection,

however, detection in western blotting is not as sensitive as electrophysiological recordings, nor does it show any functional consequence. Though in reduced amounts, STIM may have still been expressed enough to observe functionality in whole-cell recordings. Therefore, it is possible that the level of reduction by our shRNA_{STIM1/2} was not sufficient in abolishing STIM function.

This result could be influenced by many factors including the limited effectiveness of shRNA in achieving complete knockdown and/or the overexpression of visual fluorescent markers increasing cellular stress and promoting pannexin activation as part of an inflammatory response. Although several interpretations to this negative result can be inferred, ultimately, this finding led me to consider alternative candidate mechanism underlying ER stress induced pannexin activation.

4.6 Exploring alternative mechanisms of pannexin activation in neurons

A recent study demonstrating anoxia-induced pannexin currents employed Src-family kinase inhibitor PP2 to prevent pannexin currents by blocking tyrosine phosphorylation at the pannexin c-terminus mediated by NMDA-receptor activation (Weilinger et al., 2012). In this study done by the Thompson lab, upon anoxia-induced pannexin activation, initial depolarization, residual current after block and cumulative charge transfer were analyzed. Residual current after block, which represents non-pannexin mediated activity, and cumulative charge transferred were significantly reduced in PP2-treated groups compared to vehicle treated. This study was performed in a model of anoxia, a well-known model

for generating ER stress (Bodalia et al., 2012), thus providing rationale for consideration as a candidate mechanism underlying the ER stress-induced model of pannexin activation I have presented. Furthermore, recent evidence demonstrating the activation of Src-family kinases in response to ER stress (Yu and Kim, 2010) has made Src a candidate to be investigated as a mediator of ER stress induced pannexin activation.

In this experiment, pannexin currents were recorded in response to 3 μ M thapsigargin in the presence of 10 μ M PP2, an inhibitor of Src-family kinases, applied extracellularly through the bath solution. These recordings were from isolated pyramidal neurons of the mouse CA1 hippocampus region. The results show that ER stress-induced pannexin currents were still evoked in the presence of a Src-family kinase inhibitor. In accordance with the results from the Thompson lab, the same parameters were quantified and analyzed. However, there was no difference found in the peak current amplitude, residual current after block with 10 μ M lanthanum or total charge transferred across the membrane between Src-kinase inhibitor and vehicle treated groups (Fig. 3.5). This result has yet to be supported in our lab with data from phosphorylation assays to determine the effectiveness of extracellular 10 μ M PP2 in inhibiting tyrosine phosphorylation under ER stress. This discrepancy in outcomes suggests that the underlying mechanism of pannexin activation is likely to be modality specific. Although the literature suggests an overlap in activation pathways, I have demonstrated that ER stress-induced pannexin activation and anoxia-induced pannexin activation are not dependent on the same signaling molecules.

A recent study by the Ravichandran lab, published in 2010, identified yet another potential mechanisms provoking pannexin channel activation. They showed that pannexin-1 is a target for caspase 3 and 7. They identified a specific caspase-cleavage site at the C-terminus of pannexin-1. Cleavage at this site was essential for pannexin activation during apoptosis. A truncated variant of pannexin-1, truncated at its caspase-cleavage site at the C-terminus, resulted in constitutive activation whereas pharmacological inhibition of caspases 3 and 7 inhibited activation of wild type pannexin-1 (Chekeni et al., 2010). The findings of this study provided me with an alternative candidate mechanism for pannexin activation. Given that ER stress has previously been shown to lead to caspase activation (Nakagawa et al., 2000), I reasoned that activation of caspases and their subsequent cleavage of pannexin c-terminal may provide a mechanism for pannexin activation. For this experiment, I recorded pannexin currents in response to 3 μ M thapsigargin in the presence of 20 μ M zVAD-fmk, an inhibitor of caspases 3 and 7, applied intracellularly through the patch electrode, as performed by the Ravichandran lab. I recorded from isolated pyramidal neurons of the mouse CA1 hippocampus region. My preliminary observations convincingly show ER stress induced pannexin activation even in the presence of caspase inhibitor (Fig. 3.6). Although more replicates need to be done before reporting a conclusive result, it appears caspase-cleavage does not contribute to pannexin channel activation under the modality of ER stress. This result has yet to be supported in our lab with data from experiments testing caspase activity to determine the effectiveness of intracellular 20 μ M zVAD-FMK in inhibiting caspase activity during ER stress.

This finding did however reinstate the view that pannexin activation has modality specific mechanisms. This view is supported by the divergence between ER stress-induced and apoptosis-induced pannexin activation in addition to the previously described divergence from anoxia-induced pannexin activation.

4.7 Experiments for future studies

I hypothesized that pannexin channel activation during ER stress is contingent on recruitment of STIM proteins. This was primarily supported by results in HEK 293 cells where expression of STIM is necessary to reconstitute pannexin channel activation in response to thapsigargin application. In this model system, my results using a STIM deletion construct suggests that the ERM domain of STIM, which includes the CAD activating domain necessary for STIM-coupling to ORAI channels, is also necessary for pannexin channel activation by ER stress. Given this finding and considering the relatively large size (286 amino acids) of the ERM domain, a follow-up experiment would be to delete only the CAD domain (STIM1 Δ CAD), rather than the entire ERM domain, to prove that CAD alone is the region interacting with pannexin channel and that this interaction does not involve other components of the ERM.

If the result is positive (ie. Panx1 current is abolished in STIM1 Δ CAD transfected HEK cells), the next objective would be to determine whether the CAD domain of STIM1 is sufficient to activate pannexin-1 by transfecting only the CAD domain fragment (rather than full-length STIM1 with a deleted CAD domain) into HEK 293 cells and recording for constitutively active panx1 currents. A positive result in this experiment (ie. ERM

fragment is sufficient to activate panx1 channels), would provide further support for my hypothesis in the context of HEK cells where ER stress induced pannexin activation is mediated by STIM. Additionally, this positive result would show that pannexin activation is not only achieved using pharmacological ER stress inducers but also by the constitutively active form of an ER stress sensing protein. Since ERM and CAD fragments would no longer be attached to the intraluminal calcium binding E-F hands of STIM, these fragments would be useful tools in modeling constitutively active STIM. This would refute any criticism that non-specific pharmacological ER stressors may be activating other cellular processes that could contribute to the observed response.

Another interpretation of the negative electrophysiological result in the STIM-KD experiment is that STIM proteins do not play a role in neuronal ER stress induced pannexin activation. To consider this interpretation, we need a positive control to functionally confirm the knock down of STIM proteins upon lentiviral infection of shRNA_{STIM1&2}. This can be confirmed by using calcium imaging to assay for store-operated calcium entry after the shRNA knock down of STIM. Since store-operated Ca²⁺ entry is contingent on STIM proteins we should not observe any store-operated Ca²⁺ entry in shRNA_{STIM1&2} infected neurons (Liou et al., 2005; Roos et al., 2005). This result would add confidence in the negative finding resulting from STIM knockdown experiments and would allow me to reject my hypothesis of STIM-pannexin coupling in the context of neuronal ER stress induced pannexin activation. To avoid skepticism towards effectiveness of the knock down, an ideal experiment to test my hypothesis

would be to record ER stress induced pannexin activation in neurons from STIM knockout mice.

A recent study highlighting pannexin involvement in an *in vivo* model of cerebral ischemia used pannexin-1^{-/-}, pannexin-2^{-/-} and pannexin-1^{-/-}pannexin-2^{-/-} knockout mice to determine the contribution of each form to neuronal cell death. Interestingly, pannexin-1^{-/-}pannexin-2^{-/-} knockout mice demonstrated smaller infarct sizes and increased cell viability, whereas damage in pannexin-1^{-/-} and pannexin-2^{-/-} was the same as in wild-type mice (Bargiotas et al., 2011). To further my own findings, I would record ER stress induced currents from pannexin-1 knockout mice to understand whether or not there is any contribution by pannexin-2 to the aforementioned ER stress induced pannexin activation.

Furthermore, now that I have shown pannexin activation in response to A β ₄₂ treatment, a future study would be to repeat that experiment in neuronal cultures from pannexin-1 knockout mice. I would expect the constitutive current observed at the onset of the recording to be abolished. This would provide rationale for a cell death assay to investigate the relative A β ₄₂-mediated neurotoxicity in pannexin-1 knockout neurons compared to wild-type neurons. This set of experiments would add to the significance of the project by characterizing a role for pannexin channel activation in AD.

4.8 Significance of the study

The importance of my study is highlighted by the limited information on the regulation of pannexin channel activity. Despite numerous findings of novel pannexin channel function, very few studies have examined molecular mechanisms underlying pannexin activation. My findings do not refute any of the previous implications for pannexin channel involvement in various pathophysiological conditions; rather it adds another possible mechanism of activation for the field to consider. In this project, I have established ER stress as a means of activating pannexin channels, not to be misunderstood for proposing a novel function of pannexin channels. Accordingly, my finding implicates pannexin channels as a downstream mediator of the ER stress response. It is entirely possible that the pannexin activation demonstrated by other groups in various contexts including inflammation, apoptosis and hypoxia is a downstream effect of the ER stress that is associated with these conditions.

Broader implications of my study stem from the involvement of ER stress in neurodegeneration. By presenting evidence of pannexin activation in a model of Alzheimer's Disease, the contribution of pannexin channels to neurotoxicity and the disruption of the ER stress-pannexin coupling in neurodegeneration becomes a focal point for future projects.

Section 5

REFERENCES

- Ambrosi C, Gassmann O, Pranskevich JN, Boassa D, Smock A, Wang J, Dahl G, Steinem C, Sosinsky GE. (2010). Pannexin1 and Pannexin2 channels show quaternary similarities to connexons and different oligomerization numbers from each other. *J Biol Chem.* 285(32): 24420-31.
- Banhegyi G, Mandl J, Csala M. (2008). Redox-based endoplasmic reticulum dysfunction in neurological diseases. *J Neurochem.* 107(1): 20-34.
- Bao L, Locovei S, Dahl G. (2004). Pannexin membrane channels are mechanosensitive conduits for ATP. *FEBS Lett.* 572(1-3): 65-8.
- Baranova A, Ivanov D, Petrash N, Pestova A, Skoblov M, Kelmanson I, Shagin D, Nazarenko S, Geraymovych E, Litvin O, Tiunova A, Born TL, Usman N. (2004). The mammalian pannexin family is homologous to the invertebrate innexin gap junction proteins. *Genomics.* 83(4): 706-16.
- Bargiotas P, Krenz A, Hormuzdi SG, Ridder DA, Herb A, Barakat W, Penuela S, von Engelhardt J, Monyer H, SCHwaninger M. (2011). Pannexins in ischemia-induced neurodegeneration. *Proc Natl Acad Sci USA.* 108(51): 20772-7.
- Berna-Erro A, Braun A, Kraft R, Kleinschnitz C, Schuhmann MK, Stegner D, Wultsch T, Eilers J, Meuth SG, Stoll G, Nieswandt B. (2009). STIM2 regulates capacitive Ca^{2+} entry in neurons and plays a key role in hypoxic neuronal cell death. *Sci Signal.* 2(92): ra67.
- Bhalla-Gehi R, Penuela S, Churko JM, Shao Q, Laird DW. (2010). Pannexin1 and pannexin3 delivery, cell surface dynamics, and cytoskeletal interactions. *J Biol Chem.* 285(12): 9147-60.
- Bodalia A, Li H, Jackson MF. (2012) Loss of endoplasmic reticulum Ca^{2+} homeostasis: contribution to neuronal cell death during cerebral ischemia. *Acta Pharmacol Sin.* 34(1): 49-59.
- Boassa D, Ambosi C, Qiu F, Dahl G, Gaietta G, Sosinsky G. (2007). Pannexin1 channels contain a glycosylation site that targets the hexamer to the plasma membrane. *J Biol Chem.* 282(43): 31733-43.
- Brandman O, Liou J, Park WS, Meyer T. (2007) STIM2 is a feedback regulator that stabilizes basal cytosolic and endoplasmic reticulum Ca^{2+} levels. *Cell.* 131(7): 1327-39.
- Bruzzone R, Hormuzdi SG, Barbe MT, Herb A, Monyer H. (2003). Pannexins, a family of gap junction proteins expressed in the brain. *Proc Natl Acad Sci USA.* 100(23): 13644-9.

- Burdakov D, Petersen OH, Verkhratsky A. (2005). Intraluminal calcium as a primary regulator of endoplasmic reticulum function. *Cell Calcium*. 38(3-4): 303-10.
- Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, Clark SG, Ron D. (2002). IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature*. 415(6867): 92-6.
- Chefekar SM, Hoosemans JJ, Zwart R, Baas F, Scheper W. (2007). Abeta 1-42 induced mild endoplasmic reticulum stress in an aggregation-state dependent manner. *Antioxid Redox Signal*. 9(12): 2245:54.
- Chekeni FB, Elliot MR, Sandilos JK, Walk SF, Kinchen JM, Lazarowski ER, Armstrong AJ, Penuela S, Laird DW, Salvesen GS, Isakson BE, Bayliss DA, Ravichandran KS. (2010). Pannexin-1 channels mediate the “find me” signal release and membrane permeability during apoptosis. *Nature*. 467(7317): 863-7.
- Dahl G, Werner R, Levine E, Rabadan-Diehl C. (1992) Mutational analysis of gap junction formation. *Biophys J*. 62(1): 172-80.
- De Felice FG, Velasco PT, Lambert MP, Viola K, Fernandez SJ, Ferreira ST, Klein WT. (2007). Abeta oligomers induce neuronal oxidative stress through an N-methyl-D-aspartate receptor-dependent mechanism that is blocked by Alzheimer drug memantine. *J Biol Chem*. 282(15): 11590-601.
- Demuro A, Parker I, Stutzmann GE. (2010). Calcium signaling and amyloid toxicity in Alzheimer disease. *J Biol Chem*. 285(17): 12463-8.
- D’hondt C, Ponsaerts R, De Smedt H, Bultynck G, Himpens B. (2009). Pannexins, distant relative of the connexin family with specific cellular functions?. *Bioessays*. 31(9): 953-74.
- Ferreiro E, Resende R, Costa R, Oliveira CR, Pereira CM. (2006). An endoplasmic-reticulum-specific apoptotic pathway is involved in prion and amyloid-beta peptides neurotoxicity. *Neurobiol Dis*. 23(3): 669–678.
- Feske S, Gwack Y, Prakriya M, Srikanth S, Puppel SH, Tanasa B, Hogan PG, Lewis RS, Daly M, Rao A. (2006). A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature*. 441(790): 179-85.
- Galehdar Z, Swan P, Fuerth B, Callaghan SM, Park DS, Cregan SP. (2010). Neuronal apoptosis induced by endoplasmic reticulum stress is regulated by ATF4-CHOP-mediated induction of the Bcl-2 homology 3-only member PUMA. *J Neurosci*. 30(50): 16938–48.

- Gehi R, Shao Q, Laird DW. (2011). Pathways regulating the trafficking and turnover of pannexin1 protein and the role of the C-terminal domain. *J Biol Chem.* 286(31): 27639-53.
- Harama D, Koyama K, Mukai M, Shimokawa N, Miyata M, Nakamura Y. (2009). A subcytotoxic dose of subtilase cytotoxin prevents lipopolysaccharide-induced inflammatory responses, depending on its capacity to induce the unfolded protein response. *J Immunol.* 183(2): 1368–1374.
- Hawkins BJ, Irrinki KM, Mallilankaraman K, Lien YC, Wang Y, Bhanumathy CD, Subbiah R, Ritchie MF, Soboloff J, Baba Y, Kurosaki T, Joseph S, Gill DL, Madesh M. (2010). S-glutathionylation activates STIM1 and alters mitochondrial homeostasis. *J Cell Biol.* 190(3): 391–405.
- Holz WA, O'Malley KL. (2003). Parkinsonian memetics induce aspects of unfolded protein response in death of dopaminergic neurons. *J Biol Chem.* 278(21): 19367-77.
- Hoozemans JJ, Veerhuis R, Van Hasstert ES, Rozemuller JM, Baas F, Eikelenboom P, Scheper W. (2005). The unfolded protein response is activated in Alzheimer's Disease. *Acta Neuropathol.* 110(2): 165-72.
- Huang GN, Zeng W, Kim JY, Yuan JP, Han L, Muallem S, Worley PF. (2006). STIM1 carboxyl-terminus activates native SOC, I(crac) and TRPC1 channels. *Nat Cell Biol.* 8(9): 1003–10.
- Imai Y, Soda M, Inoue H, Hattori N, Mizuno Y, Takahashi R. (2001). An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. *Cell.* 105(7): 891-902.
- Jiang H, Zhu AG, Mameczur M, Falck JR, Lerea KM, McGiff JC. (2007). Stimulation of rat erythrocyte P2X7 receptor induces the release of epoxyeicosatrienoic acids. *Br J Pharmacol.* 151(7): 1033-40.
- Kaneko M, Koike H, Saito R, Kitamura Y, Okuma Y, Nomura Y. (2010). Loss of HRD1-mediated protein degradation causes amyloid precursor protein accumulation and amyloid- β generation. *J Neurosci.* 30(11): 3924–32.
- Katayama T, Imaizumi K, Honda A, Yoneda T, Kudo T, Takeda, M, Mori K, Rozmahel R, Fraser P, George-Hyslop PS, Tohyama M. (2001). Disturbed activation of endoplasmic reticulum stress transducers by familial Alzheimer's disease-linked presenilin-1 mutations. *J Biol Chem.* 276(46): 43446–43454.
- Kohno K, Higuchi T, Ohta S, Kohno K, Kumon Y, Sakaki S. (1994). Neuroprotective nitric oxide synthase inhibitor reduced intracellular calcium accumulation following transient global ischemia in the gerbil. *Neurosci Lett.* 224(1): 17-20.

- Lee do Y, Lee KS, Lee HJ, Kim do H, Noh YH, Yu K, Jung HY, Lee SH, Lee JY, Youn YC, Jeong Y, Kim DK, Lee WB, Kim SS. (2010). Activation of PERK signaling attenuates Abeta-mediated ER stress. *PLoS. One.* 5(5): e10489.
- Lehrman MA. (2001). Oligosaccharide-based information in endoplasmic reticulum quality control and other biological systems. *J Biol Chem.* 276(12): 8623-6.
- Liou J, Kim ML, Heo WD, Jones JT, Myers JW, Ferrell JE Jr, Meyer T. (2005). STIM is a Ca^{2+} sensor essential for Ca^{2+} -store-depletion-triggered Ca^{2+} influx. 15(13): 1235–41.
- Locovei S, Bao L, Dahl G. (2006). Pannexin-1 in erythrocytes: Function without a gap. *Proc Natl Acad Sci USA.* 103(20): 7655-9.
- Ma W, Hui H, Pelegrin P, Surprenant A. (2009). Pharmacological characterization of pannexin-1 currents expressed in mammalian cells. *J Pharmacol Exp Ther.* 328(2): 409-18.
- Ma W, Compan V, Zheng W, Martin E, North RA, Verkhratsky A, Surprenant A. (2012). Pannexin-1 forms an anion-selective channel. *Pflugers Arch.* 463(4): 585-92.
- MacDonald JF, Mody I, Salter MW. (1989). Regulation of N-methyl-D-aspartate receptors revealed by intracellular dialysis of murine neurones in culture. *J Physiol.* 414; 17–34.
- MacVicar BA, Thompson RJ. (2010). Non-junction functions of pannexin-1 channels. *Trends Neurosci.* 33(2): 93-102.
- Mc Donald JM, Savva GM, Brayne C, Welzel AT, Forster G, Shankar GM, Selkoe DJ, Ince PG, Walsh DM. (2010). The presence of sodium dodecyl sulphate-stable Abeta dimers is strongly associated with Alzheimer-type dementia. *Brain.* 133(5): 1328-41.
- McLean CA, Cherny RA, Fraser FW, Fuller SJ, Smith MJ, Beyreuther K, Bush AI, Masters CL. (1999). Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's Disease. *Ann Neurol.* 46(6): 860-6.
- Michalak M, Robert Parker JM, Opas M. (2002). Ca^{2+} signaling and calcium binding chaperones of the endoplasmic reticulum. *Cell Calcium.* 32(5-6): 269-78.
- Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J. (2000). Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid beta. *Nature.* 403(6765): 98-103.
- Nguyen HN, Wang C, Perry DC. (2002). Depletion of intracellular calcium stores is toxic to SH-SY5Y neuronal cells. *Brain Res.* 924(2): 159-66.

- Orrenius S, Zhivotovsky B, Nicotera P. (2003). Regulation of cell death: the calcium-apoptosis link. *Nat Rev Mol Cell Biol.* 4(7): 552-65.
- Penuela S, Bhalla R, Gong XQ, Cowan KN, Celetti SJ, Cowan BJ, Shao Q, Laird DW. (2007). Pannexin 1 and pannexin 3 are glycoproteins that exhibit distinct characteristics from the connexin family of gap junction proteins. *J Cell Sci.* 120(21): 3772-83.
- Penuela S, Bhalla R, Nag K, Laird DW. (2009). Glycosylation regulates pannexin intermixing and cellular localization. *Mol Biol Cell.* 20(20): 4313-23.
- Phelan P, Starich TA. (2001). Innexins get into the gap. *Bioessays.* 23(5): 388-96.
- Pirot P, Eizirik DL, Cardozo AK. (2006). Interferon-gamma potentiates endoplasmic reticulum-induced death by reducing pancreatic beta cell defence mechanisms. *Diabetologia.* 49(6): 1229-36.
- Puthalakath H, O'Reilly LA, Gunn P, Lee L, Kelly PN, Huntington ND, Hughes PD, Michalek EM, McKimm-Breschkin J, Motoyama N, Gotoh T, Akira S, Bouillet P, Strasser A. (2007). ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell.* 129(7): 1337-49.
- Putney JW. (1986). A model for receptor-regulated calcium entry. *Cell Calcium.* 7(1):1-12.
- Ransford GA, Fregien N, Qiu F, Dahl G, Conner GE, Salathe M. (2009) Pannexin 1 contributes to ATP release in airway epithelia. *Am J Respir Cell Mol Biol.* 41(5): 525-34.
- Ricobaraza A, Cuadrado-Tejedor M, Marco S, Perez-Otano I, Garcia-Osta A. (2010). Phenylbutyrate rescues dendritic spine loss associated with memory deficits in a mouse model of Alzheimer disease. *Hippocampus.* 22(5): 1040-50.
- Roos J, DiGregorio PJ, Yeromin AV, Ohlsen K, Lioudyno M, Zhang S, Safrina O, Kozak JA, Wagner SL, Cahalan MD, Velicelebi G, Stauderman KA. (2005). STIM1, an essential and conserved component of store-operated Ca^{2+} channel function. *J Cell Biol.* 169(3): 435–45.
- Ryu EJ, Harding HP, Angelastro JM, Vitolo OV, Ron D, Greene LA. (2002). Endoplasmic reticulum stress and the unfolded protein response in cellular models of Parkinson's Disease. *J Neurosci.* 22(24); 10690-8.
- Sabbioni S, Barbanti-Brodano G, Croce CM, Negrini M. (1997). GOK: a gene at 11p15 involved in rhabdomyosarcoma and rhabdoid tumor development. *Cancer Res.* 57(20): 4493–7.

- Sandilos JK, Chiu YH, Chekeni FB, Armstrong AJ, Walk SF, Ravichandran KS, Bayliss DA. (2012). Pannexin1, an ATP release channel, is activated by caspase cleavage of its pore-associated C-terminal. *J Biol Chem.* 287(14): 11303-11.
- Seyb KI, Ansar S, Bean J, Michaelis ML. (2006). Beta-amyloid and endoplasmic reticulum stress responses in primary neurons: effects of drugs that interact with the cytoskeleton. *J Mol Neurosci.* 28(2): 111–123.
- Shiraishi T, Yoshida T, Nakata S. (2005). Tunicamycin enhances tumor necrosis factor-related apoptosis-inducing ligand induced apoptosis in human prostate cancer cells. *Cancer Res.* 65(14): 6364–70.
- Soboloff J, Madesh M, Gill DL. (2011). Sensing cellular stress through STIM proteins. *Nat Chem Biol.* 7(8): 488-92.
- Soboloff J, Rothberg BS, Madesh M, Gill DL. (2012). STIM proteins: dynamic calcium signal transducers. *Nat Rev Mol Cell Biol.* 13(9): 549-65
- Stathopulos PB, Li GY, Plevin MJ, Ames JB, Ikura M. (2006). Stored Ca^{2+} depletion-induced oligomerization of stromal interaction molecule 1 (STIM1) via the EF-SAM region: An initiation mechanism for capacitive Ca^{2+} entry. *J Biol Chem.* 281(47): 35855–62.
- Stathopulos PB, Zheng L, Li GY, Plevin MJ, Ikura M. (2008). Structural and mechanistic insights into STIM1-mediated initiation of store-operated calcium entry. *Cell.* 135(1): 110–22.
- Stutzmann GE, Mattson MP. (2011). Endoplasmic reticulum Ca^{2+} handling in excitable cells in health and disease. *Pharmacol Rev.* 63(3): 700-27.
- Tajiri S, Oyadomari S, Yano S, Morioka M, Gotoh T, Hamada JI, Ushio Y, Mori M. (2004). Ischemia-induced neuronal cell death is mediated by the endoplasmic reticulum stress pathway involving CHOP. *Cell Death Differ.* 11(4): 403–15.
- Takahasi K, Niidome T, Akaike A, Kihara T, Sugimoto H. (2009). Amyloid precursor protein promotes endoplasmic reticulum stress-induced cell death via C/EBP homologous protein-mediated pathway. *J Neurochem.* 109(5): 1324-37.
- Tobmal B, Weeraratne AT, Denmeade SR, Isaacs JT. (2000). Thapsigargin induces a calmodulin/calcineurin-dependent apoptotic cascade responsible for the death of prostatic cancer cells. *Prostate.* 43(4): 303-17.
- Townsend M, Shankar GM, Mehta T, Walsh DM, Selkoe DJ. (2006). Effects of secreted oligomer of beta-amyloid-protein on hippocampal synaptic plasticity: a potent role for trimmers. *J Physiol.* 572(2): 477-92.

- Troncoso JC, Martin LJ, G Dal Forno, Kawas CH. (1996). Neuropathology in controls and demented subjects from the Baltimore Longitudinal Study of Aging. *Neurobiol Aging*. 17(3): 365-71.
- Truettner JS, Hu K, Liu CL, Dietrich WD, Hu B. (2009). Subcellular stress response and induction of molecular chaperones and folding proteins after transient global ischemia in rats. *Brain Res*. 1249: 9-18.
- Unterberger U, Hoftberger R, Gelpi E, Flicker H, Budka H, Voigtlander T. (2006). Endoplasmic reticulum stress features are prominent in Alzheimer Disease but not in prion diseases in vivo. *J Neuropathol Exp Neurol*. 65(4): 348-57.
- Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D. (2000). Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science*. 287(5453): 664-6.
- Verkhatsky A, Petersen OH. (2002). The endoplasmic reticulum as an integrating signaling organelle: from neuronal signaling to neuronal death. *Eur J Pharmacol*. 447(2-3): 141-54.
- Wang Y, Deng X, Mancarella S, Hendron E, Eguchi S, Soboloff J, Tang XD, Gill DL. (2010). The calcium store sensor, STIM1, reciprocally controls Orai and CaV1.2 channels. *Science*. 330(6000): 105-9.
- Weilinger NL, Tang PL, Thompson RJ. (2012). Anoxia-induced NMDA receptor activation opens pannexin channels via Src family kinases. *J Neurosci*. 32(36): 12579-88.
- Wiley JC, Pettan-Brewer C, Ladiges WC. (2011). Phenylbutyric acid reduces amyloid plaques and rescues cognitive behavior in AD transgenic mice. *Aging Cell*. 10(3): 418-28.
- Xiao B, Coste B, Mathur J, Patapoutian A. (2011). Temperature-dependent STIM1 activation induces Ca^{2+} influx and modulates gene expression. *Nat Chem Biol*. 7(6): 351-8.
- Xing H, Azimi-Zonooz A, Shuttleworth CW, Connor JA. (2004). Caffeine releasable stores of Ca^{2+} show depletion prior to the final steps in delayed CA1 neuronal death. *J Neurophysiol*. 92(5): 2960-7.
- Yu SM, Kim SJ. (2010). Endoplasmic reticulum stress (ER-stress) by 2-deoxy-D-glucose (2DG) reduces cyclooxygenase-2 (COX-2) expression and N-glycosylation and induces a loss of COX-2 activity via a Src kinase-dependent pathway in rabbit articular chondrocytes. *Exp Mol Med*. 42(11): 777-86.

- Zhang SL, Yeromin AV, Zhang XH, Yu Y, Safrina O, Penna A, Roos J, Stauderman KA, Cahalan MD. (2006). Genome-wide RNAi screen of Ca^{2+} influx identifies genes that regulate Ca^{2+} release-activated Ca^{2+} channel activity. *Proc Natl Acad Sci USA*. 103(24): 9457-62.
- Zhou Y, Mancarella S, Wang Y, Yue C, Ritchie M, Gill DL, Soboloff J. (2009). The short N-terminal domains of STIM1 and STIM2 control the activation kinetics of Orai1 channels. *J Biol Chem*. 284(29): 19164–8.
- Zoidl G, Petrasch-Parwez E, Ray A, Meier C, Bunse S, Habbes HW, Dahl G, Dermietzel R. (2007). Localization of the pannexin1 protein at postsynaptic sites in the cerebral cortex and hippocampus. *Neuroscience*. 146(1): 9-16.

Appendices

Appendix A: Supplementary Figures

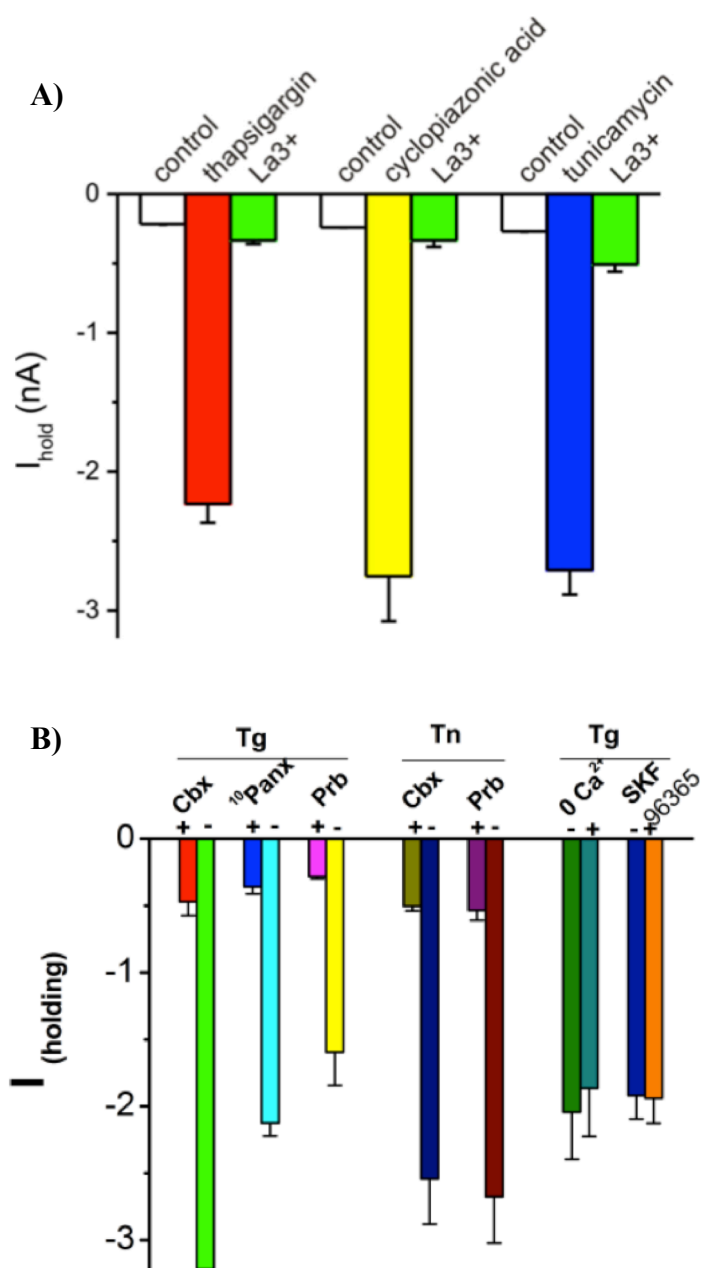


Figure S1. Unpublished lab data illustrating A) various pharmacological ER stress inducers activating La³⁺ sensitive currents in neurons. B) Pharmacological profile of ER stress induced currents rule-in pannexin channels and rule-out other non-selective cation channels in neurons.

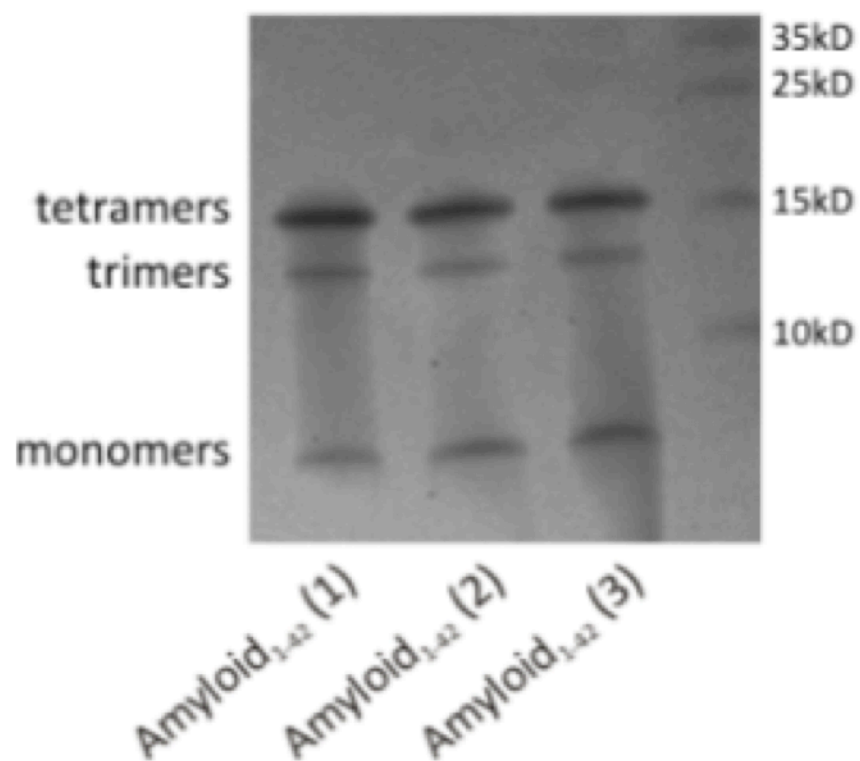


Figure S2. Unpublished lab data. Western blot characterization of Aβ₄₂ oligomers composed as trimers and tetramers.

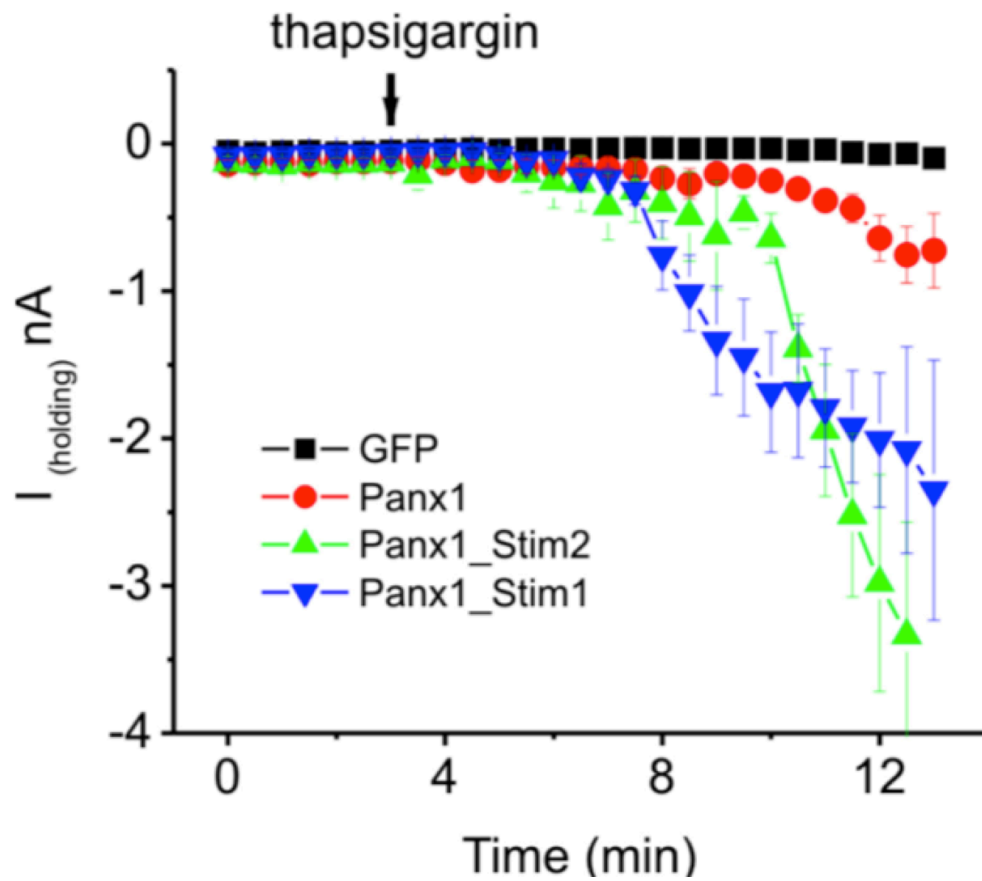
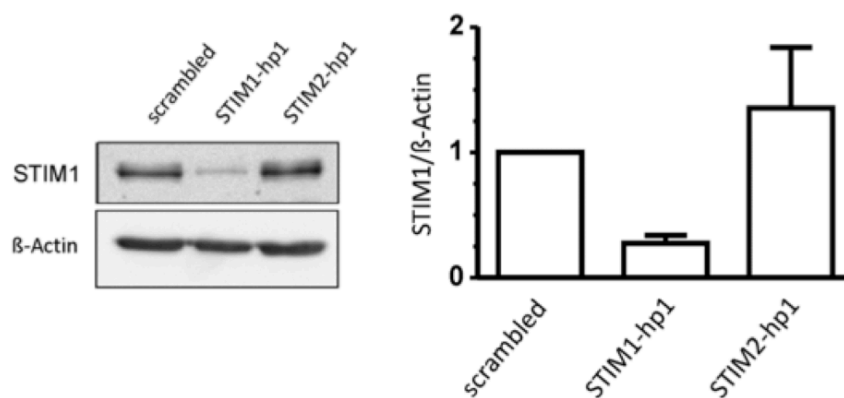


Figure S3. Unpublished lab data illustrating that complete reconstitution of thapsigargin-evoked current in HEK cells requires co-transfection of pannexin-1 and either STIM1 or STIM2.

STIM1 knockdown by RNAi



STIM2 knockdown by RNAi

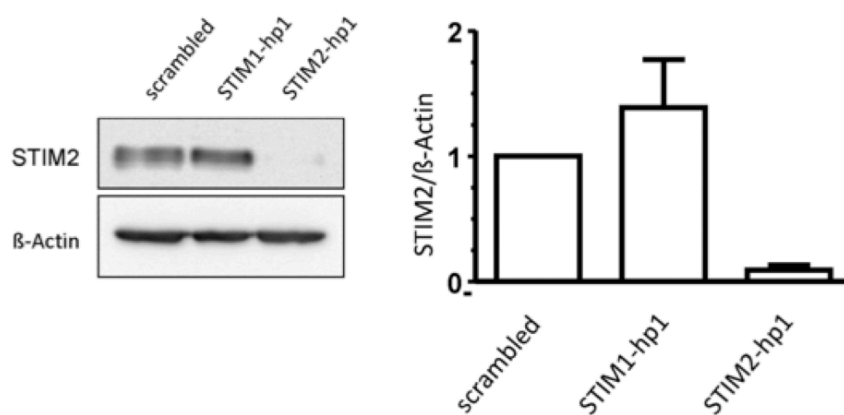


Figure S4. Unpublished lab data demonstrating the effectiveness of RNAi knockdown of STIM1 and STIM2, following lentiviral infection in neurons, by Western blot and quantified densitometrically (normalized to β -actin).

Appendix B: Curriculum Vitae

Ankur Bodaila

Education

Western University: Masters of Science in Pharmacology and Toxicology

Sept. 2011- Sept. 2013

Thesis: ER stress coupled pannexin activation via STIM proteins

Supervisors: Drs. Michael F. Jackson and John F. MacDonald

Western University: Bachelor of Sciences (Honors Specialization in Medical Sciences)

Sept. 2007 – Apr. 2011

Scholarships and Awards

First Place – Neuroscience & Mental Health – London Health Research Day 2013

Mar. 2013

- Poster presentation titled “ER stress induced pannexin activation via STIM coupling”

Western University Graduate Thesis Research Award – Travel Scholarship

Jan. 2013

- Value: \$500 Duration: Jan. 2013 – Aug. 2013

Alzheimer’s Foundation of London and Middlesex Scholarship – M.Sc. Award

Nov. 2012

- Value: \$12 500 Duration: Sept. 2012 – Aug. 2013

First Place – Neuroscience – Dept. of Physiology & Pharmacology Research Day

Nov. 2012

- Poster presentation titled “ER stress induced pannexin activation via STIM coupling”

Teaching Experience

Western University – Department of Physiology & Pharmacology

2012-2013 Mammalian Physiology 3130Y – Course Coordinator: Thomas Stavraky

2011-2012 Mammalian Physiology 3130Y – Course Coordinator: Thomas Stavraky

Publications

Bodalia A, Li H, Jackson MF (2012) Loss of endoplasmic reticulum Ca²⁺ homeostasis: contribution to neuronal cell death during cerebral ischemia. *Acta Pharmacologica Sinica*. 34(1): 49-59. Review.